Zinc(II) Binding to Apo-(Bovine Erythrocyte Superoxide Dismutase)

By ANTHONY E. G. CASS and H. ALLEN O. HILL*

Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, U.K.,

and JOSEPH V. BANNISTER and WILLIAM H. BANNISTER

Department of Physiology and Biochemistry, University of Malta, Msida, Malta

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The binding of zinc(II) ions to apo-(bovine erythrocyte superoxide dismutase) was studied by ^1H n.m.r. spectroscopy. Two zinc(II) ions bind to each subunit of the apoenzyme, and the first has a binding constant at least an order of magnitude larger than the second. The nature of the spectral changes that occur on binding the first zinc(II) ion are interpreted in terms of a change in the structure of the protein around the active site to one very similar to that of the holoenzyme, thus pre-forming the second zinc(II) binding site. The binding of the second zinc(II) ion effects changes in the environment of only those residues involved in its co-ordination.

The cytosol of eukaryotes contains a copper–zinc enzyme known as superoxide dismutase (EC 1.15.1.1), which is believed to be responsible for scavenging the superoxide radical anion by enhancing the rate of its dismutation (Fridovich, 1975; Michelson *et al.*, 1977).

The enzyme isolated from bovine erythrocytes has been the most intensively studied, and both the complete amino acid sequence (Steinman *et al.*, 1974) and the X-ray structure at 0.3 nm (3 Å) resolution are known (Richardson *et al.*, 1975).

Reconstitution experiments have shown (Beem *et al.*, 1974) that although copper is absolutely required for the enzymic activity, the protein with 1 copper ion per subunit is only partially active and a second metal is required for the full development of the native enzyme properties. This second metal may be zinc, as it is in the native enzyme, or it may be cadmium, cobalt, mercury or a second copper ion. In these cases the second metal ion is assumed (Beem *et al.*, 1974) to occupy the same site as zinc in the native enzyme.

The X-ray-diffraction studies have revealed (Richardson *et al.*, 1975) an intimate relationship between the two metal-binding sites, as suggested by earlier spectroscopic studies (Fee & Gaber, 1972). The copper and zinc atoms are approx. 0.6 nm (6 Å) apart, with the imidazole ring of histidine-61 interposed between them (Richardson *et al.*, 1975). The role of the metal in the second (zinc) site has been suggested to be a structural one. This is partially borne out by the greater resistance of the zinc- or

mercury-treated apoenzyme to chemical or thermal denaturation (Forman & Fridovich, 1973). We have previously presented (Cass *et al.*, 1977a) a high-resolution ^1H n.m.r. spectroscopic study of bovine erythrocyte superoxide dismutase in which we proposed a number of assignments to specific amino acid residues. During the course of this work we noted characteristic differences between the apoenzyme and the Cu(I)–Zn(II) enzyme. These were ascribed to differences in the structures of the two forms. The present work describes a systematic study of zinc binding to the apoenzyme.

Experimental

Superoxide dismutase from bovine erythrocytes was isolated by the method of Bannister *et al.* (1971), and the apoprotein prepared by the method of Weser & Hartman (1971); excess EDTA was removed by dialysis against NaClO_4 (Fee, 1973).

ZnSO_4 (99.999%) was obtained from Hazelwood Chemicals (Staines, Middx., U.K.). $^2\text{H}_2\text{O}$ (99.8% ^2H) and ^2HCl were from Merck, Sharpe and Dohme (Montreal, Que., Canada), NaO^2H solution was from CIBA (Basel, Switzerland). All other reagents were analytical grade and the water was doubly distilled. Solutions for ^1H n.m.r. (150 mg/ml) were prepared in $^2\text{H}_2\text{O}$ containing 1 M NaCl and 20 mM-sodium phosphate. The protein solutions were exchanged in $^2\text{H}_2\text{O}$ and freeze-dried two or three times before the final solution was prepared for ^1H n.m.r. spectroscopy. The pH of the solutions was adjusted with 100 mM- NaO^2H or 100 mM- ^2HCl and the pH is quoted as direct meter readings, pH^* , uncorrected for the deuterium isotope effect.

* To whom reprint requests should be addressed.

Fig. 1. ^1H n.m.r. spectra of apo-(bovine erythrocyte superoxide dismutase) in 1M-NaCl and 20mM-phosphate in $^2\text{H}_2\text{O}$ in the presence of different amounts of zinc at pH* 6.3 and a temperature of 40°C

(a) No zinc; (b) 0.5mol of Zn(II) per mol of subunit; (c) 1 mol of Zn(II) per mol of subunit; (d) 1.5mol of Zn(II) per mol of subunit; (e) 2mol of Zn(II) per mol of subunit. The lettering is as described in the text, except that N is the resonance from the N-terminal N-acetyl group and DSS is a resonance from the internal standard sodium 2,2-dimethyl-2-silapentanesulphonate. The inset is an expanded portion of the low-field region.

^1H n.m.r. spectra were recorded at 270 MHz in the Fourier transform mode as previously described (Cass *et al.*, 1977a), and convolution difference spectra were calculated by the method of Campbell *et al.* (1973). The peak corresponding to HO^2H was suppressed by using a long-gated pulse before the measuring pulse (Campbell *et al.*, 1977). All chemical shifts are quoted in p.p.m. downfield of internal 2,2-dimethyl-2-silapentane-5-sulphonate. Protein concentrations were determined either by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma, twice crystallized) as a standard, or from the ϵ_{258} for the apoprotein (taken as $3670\text{M}^{-1}\cdot\text{cm}^{-1}$; Weser *et al.*, 1972). Zinc was determined by atomic absorption spectroscopy by using a Perkin-Elmer model 303 instrument.

Reconstitution of the apoprotein in the n.m.r. tube was by addition of $10\mu\text{l}$ portions of 100mM- ZnSO_4 from a glass micropipette. Reconstitution was also carried out under 'dilute' conditions by dissolving the apoprotein in $^2\text{H}_2\text{O}$ (10ml, 8mg/ml) and adding a single stoichiometric amount of ZnSO_4 in 1ml of $^2\text{H}_2\text{O}$. This solution was incubated at 4°C for 24h and then freeze-dried. Changes in intensity of peaks

were obtained by measuring peak heights in the convolution difference spectra.

Results and Discussion

The effect of adding Zn(II) ions to the apoenzyme causes distinct changes throughout the whole spectrum, as shown in the convolution difference spectra of apo-(superoxide dismutase) (Fig. 1a) and the enzyme reconstituted with 0.5 (Fig. 1b) and 1mol of zinc (Fig. 1c) per 15600-dalton subunit. It is apparent from these three spectra that the Zn(II) ions are in 'slow exchange' between environments: bound to the protein and free in solution. Note, for example, the disappearance of peak (m) and the appearance of peaks (n) and (o). This means that the rate of exchange of a Zn(II) ion between its site on the protein and the bulk solution is so slow that the spectrum for 0.5mol of zinc/mol of subunit is a superposition of the spectra of the apoenzyme and that for 1mol/mol of subunit. Similarly on the addition of further amounts of zinc, as illustrated by Figs. 1(d) and 1(e), which show the spectra with 1.5 and 2mol of zinc/mol of subunit, the second

Fig. 2. ^1H n.m.r. spectrum of $\text{Cu(I)}\text{-Zn(II)}$ bovine erythrocyte superoxide dismutase under the same conditions as those described in Fig. 1

The lettering is also as in Fig. 1, except that 1-6 are resonances from histidine C-2 protons as described in Cass *et al.* (1977a). The inset is an expanded portion of the low-field region.

Zn(II) ion/subunit is also in slow exchange. Resonances associated with the binding of the second Zn(II) ion do not appear until nearly all (i.e. >90%) of the first Zn(II) ion is bound, implying that the binding of zinc at its second site must have a binding constant smaller by at least a factor of 10 than binding at its first site. We can now turn to a more detailed interpretation of metal ion binding. For this it is convenient to consider the aromatic and aliphatic regions of the spectrum separately.

Aliphatic region

This is the region of the spectrum from 0 to 4.5 p.p.m., and the spectral pattern reflects the orientation of the methyl, methylene and methine protons with respect to other groups that provide the immediate magnetic environment that determines the chemical shift of particular protons. If the positions of groups around a given proton change then so will the latter's chemical shift. This means that the aliphatic region of the spectrum provides an overall view of the protein conformation. Furthermore the resonances occurring upfield of the main aliphatic peak are methyl groups shifted by virtue of the ring currents of aromatic residues (Dwek, 1973). This shift depends

on the relative orientation of the methyl group and the aromatic ring and is thus sensitive to more local changes in structure.

If we compare the spectra in Figs. 1(a), 1(c) and 2 we can see that addition of one Zn(II) ion per subunit to the apoenzyme causes its spectrum to change, in the aliphatic region at least, to one very similar to that of the $\text{Cu(I)}\text{-Zn(II)}$ protein (Fig. 2). The addition of a second Zn(II) ion causes very little further change (Fig. 1e). We can interpret this in terms of the first Zn(II) ion binding to form the structure of the holoenzyme, and pre-forming a site at which the second Zn(II) ion can bind without further alteration of the overall molecular architecture. The ^1H n.m.r. spectra of samples prepared by reconstitution under dilute conditions are identical with those prepared by using more concentrated solutions in the n.m.r. tube.

Aromatic region

In the aromatic region (5.5-9.5 p.p.m.) of the spectrum of the apoprotein, assignments have been made to the C-2 proton resonances of histidine-19 (peak a), histidine-41 (peak b) and the six histidine residues involved in metal co-ordination (peaks c-h)

Fig. 3. Variation of intensity of representative resonances in the aromatic region of the ^1H n.m.r. spectrum of apo-(bovine erythrocyte superoxide dismutase) with increasing concentrations of Zn(II) ion

This diagram illustrates one example of each of the five types of behaviour observed in the course of the zinc titration. The roman numerals refer to Table 1.

(Cass *et al.*, 1977a). The assignment of the ring protons of tyrosine-108 in the Cu(I)-Zn(II) and Cu(II)-Zn(II) holoenzymes had also been made. We have assigned tyrosine-108 resonances in the spectrum of the apoenzyme by using the spin-echo technique previously described for the holoenzyme (Cass

et al., 1977a). These resonances are labelled Y1 and Y2 in the spectra. A number of resonances corresponding to the C-4 protons of histidine residues can also be resolved, although no detailed assignments have been made. In the following we will limit ourselves to a discussion of the results for the C-2

Fig. 4. ^1H n.m.r. spectra of the aromatic region of monozinc superoxide dismutase as a function of pH^* (a) $\text{pH}^* = 5.8$; (b) $\text{pH}^* = 8.7$; (c) after returning to $\text{pH}^* 5.8$. All other conditions are as described in Fig. 1.

protons; the C-4 protons have qualitatively similar behaviour, although, as they overlap with the main aromatic envelope, interpretation is more difficult.

On addition of the first Zn(II) ion the intensities of resonances (c)–(g) decrease and new resonances, labelled (p)–(s) in Figs. 1(b) and 1(c), appear. At pH* 6.3 there is no apparent change in intensity of peaks (a), (b) and (h). If the reconstitution is repeated at pH* 5.8 then again there is no apparent change in the intensity of peaks (a) and (b), but peak (h), which has now shifted downfield, decreases in intensity and a peak now appears and increases in intensity at the position occupied by peak (h) in the spectrum of the apoprotein at pH* 6.3 and by peak (r) in that of the mono-zinc protein. This behaviour is consistent with the proposal that the C-2 protons of histidine-19 (peak a) and histidine 41 (peak b) are unaffected by ligation of the first zinc ion, but the histidine C-2 protons (peaks c–h), previously assigned as copper/zinc ligands, are perturbed by being in different chemical (magnetic) environments. Similarly the resonances Y1 and Y2 corresponding to the ring protons of tyrosine-108 are not perturbed on binding of the first Zn(II) ion.

In contrast with the aliphatic region, the aromatic portion of the spectrum shows further changes on binding of the second zinc(II) ion; this might be expected as binding would affect the chemical shifts of the C-2 protons by a direct, through-bond effect. Of the six presumed C-2-proton resonances of the mono-zinc enzyme, three, (p), (q), (s), lose intensity, whereas three, (a), (b), (r), are unaffected. Of these, two (a and b), are associated with non-ligands (see above). The three C-2 proton resonances that do lose intensity are replaced by two at different chemical shifts, (t) and (u). On the basis of this we can tentatively assign the resonances of the mono-zinc enzymes as follows: (p), (q), (s), second-binding-site ligands; (r), first-binding-site ligand; (a), (b) non-ligand histidines-19 and -41. These results are summarized in Table 1 and Fig. 3.

pH effects on mono-zinc enzyme*

We have previously discussed (Cass *et al.*, 1977a) how a differentiation between a co-ordinated and non-co-ordinated histidine residue could be made on the basis of the pH* dependence of C-2 and C-4 proton chemical shifts. It therefore seemed reasonable to expect that the non-co-ordinated histidine resonances in the mono-zinc enzyme could be detected by varying the pH*, and observing the resulting change in chemical shift. On increasing the pH* from 5.8 the resonance associated with the C-2 proton of histidine-19 (peak a) showed a normal upfield shift and that associated with histidine-41 (peak b) did not shift, but slowly lost intensity, as had been previously observed (Cass *et al.*, 1977b). Of the remaining

histidine residues, only peak (r) was unaffected; the rest showed broadening and loss of intensity. This loss of intensity was reversible on lowering the pH*, except for resonance (p), as is illustrated in Fig. 4. Such behaviour is typical of an 'intermediate exchange' process (Dwek, 1973). The irreversible loss of intensity of resonance (p) may be due to concomitant deuteration at C-2 (Markley & Cheung, 1973). This behaviour is summarized in Table 1. This exchange process does not involve a gross structural change, as the observed broadening is confined to three histidine C-2 proton resonances and is not general throughout the spectrum. In the mono-zinc, two-zinc and copper(I)–zinc(II) enzyme only six, five and six resonances respectively assigned to C-2 protons can be observed in the ^1H n.m.r. spectrum. As two of these resonances can be assigned to the non-ligand histidine residues, this means that two or three of the ligand histidine C-2 protons are not detected. One reason for this may be that they are shifted out of the expected spectral region. A number of cases are known where it has not been possible to detect all the histidine residues in the n.m.r. spectrum of a metalloprotein (Campbell *et al.*, 1975). However, the unusual pH* behaviour of the mono-zinc enzyme suggests that the reason that two histidine residues are not observed in the native reduced enzyme may be due to exchange broadening between two or more fluxional states of the active site, related to the protonation of the interposed histidine-61. During the course of the enzymic reaction the ability of the zinc site to change structure may be important and related to the rapid-proton-transfer step (McAdam *et al.*, 1977).

Extent of structural change on binding the first zinc atom

We have shown above how the ^1H n.m.r. spectra of apoenzyme, mono-zinc and Cu(I)–Zn(II) superoxide dismutase are different and how this difference reflects changes in structure. Furthermore we have shown that binding of a single zinc ion per subunit changes the spectrum of the apoenzyme to make it very similar to that of the Cu(I)–Zn(II) enzyme and have suggested that this is due to the effect of zinc in folding the protein into a native conformation. The question that still remains, however, is how great is this structural modification? Earlier studies of the circular-dichroism spectra suggested that the conformations of the apo- and holo-enzymes were the same (Wood *et al.*, 1971).

These two observations can be reconciled by the following argument. Comparison of the ^1H n.m.r. spectra for the mono-zinc and apoenzyme reveals that only some 20% of the protons are in different environments, i.e. the structural change is only limited. Furthermore, circular dichroism is only

Table 1. *Effect of pH* and Zn(II) titration on histidine and tyrosine proton resonances*

The Table summarizes the pH*- and Zn(II)-titration behaviour of histidine C-2 and tyrosine ring proton resonances in the apoproteins and mono- and two-zinc proteins. Column 1 contains the resonances studied in each of the three, modifications. The roman numerals in column 2 refer to the type of Zn(II) titration behaviour illustrated in Fig. 3. Column 3 includes the effects of pH described in this paper as well as those previously discussed in Cass *et al.* (1977a,b), and column 4 shows the tentative assignments from this and previous work.

Resonance	Zn(II) titration behaviour (Fig. 3)	-pH* titration behaviour	Assignment
(1) Apoprotein			
(a)	I	Freely titrates†	His-19†
(b)	I	Non-titrating. Deuterates at C-2†	His-41†
(c)	IV	Freely titrates†	Metal ligand†
(d)	IV	Freely titrates†	Metal ligand†
(e)	IV	Freely titrates†	Metal ligand†
(f)	IV	Freely titrates†	Metal ligand†
(g)	IV	Freely titrates†	Metal ligand†
(h)	IV	Freely titrates†	Metal ligand†
Y1	I	—	Tyr-108
Y2	I	—	Tyr-108
(2) Mono-zinc protein			
(a)	I	Freely titrates	His-19
(b)	I	Non-titrating. Deuterates at C-2	His-41
(p)	V	Non-titrating. Loses intensity irreversibly as pH increases	Copper ligand
(q)	V	Non-titrating. Loses intensity reversibly as pH increases	Copper ligand
(r)	II	Non-titrating.	Zinc ligand
(s)	V	Non-titrating. Loses intensity reversibly as pH increases	Copper ligand
Y1	I	—	Tyr-108
Y2	I	—	Tyr-108
(3) Two-zinc protein			
(a)	I		His-19
(b)	I		His-41
(r)	II		Zinc ligand
(t)	III		Copper ligand
(u)	III		Copper ligand
Y1	I		Tyr-108
Y2	I		Tyr-108

† See Cass *et al.* (1977a,b).

responsive to regular secondary structure, and in this case the eight-stranded beta-barrel (Richardson *et al.*, 1975) and those groups known to be in this barrel, histidine-19, and -41, show no perturbation on ligand binding. Tyrosine-108, although not in the beta-barrel, is distant from the metal-binding site. Therefore we suggest that the main structural element of superoxide dismutase is the same in both apo- and holo-enzyme. The role of the zinc is thus to organize the coil structure around the active site to that of the holo-protein.

Conclusions

Binding of zinc to apo-(superoxide dismutase) occurs in two distinct steps. The binding of one Zn(II) ion per subunit changes the structure in the

active-site region of the protein from that typical of the apo- to that typical of holo-protein. This binding of the first zinc ion thus pre-forms a second zinc-binding site. The binding of zinc at both sites is tight, although the binding constant for the first zinc ion is at least an order of magnitude greater than for the second. These binding sites are close and we assume that the first is the native zinc-binding site and the second is that which normally binds copper.

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