Investigation of Human Erythrocyte Superoxide Dismutase by ^1H Nuclear-Magnetic-Resonance Spectroscopy

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The 270 MHz ^1H n.m.r. spectra of the Cu(II)/Zn(II), Cu(I)/Zn(II) and apo- forms of human erythrocyte superoxide dismutase (EC 1.15.1.1) are reported. Resonances are assigned to the C-2 and C-4 protons of histidine residues in the active site, and it is suggested that five or six histidine residues serve as ligands to the metal ions in each sub-unit of the enzyme. The remaining assigned resonances are associated with histidine-41, N-terminal N-acetyl group, histidine-108 and cysteine-109. A comparison of the n.m.r. spectra of human and bovine superoxide dismutases suggests significant structural homology.

The superoxide dismutases (McCord & Fridovich, 1969) are effective catalysts for the disproportionation of the superoxide ion to H_2O_2 and O_2 :

The vast majority of studies on superoxide dismutases have been made on the enzyme from bovine erythrocytes. This enzyme, which has been more fully characterized than others, contains 151 amino acid residues, 2 g-atoms of copper and 2 g-atoms of zinc per molecule and a mol.wt. of about 32 000. The complete amino acid sequence (Steinman *et al.*, 1974) and the crystal structure to 0.3 nm resolution (Richardson *et al.*, 1975*a,b*) have been published. The enzyme has also been subjected to investigation by a variety of physicochemical techniques. We have investigated the high-resolution ^1H n.m.r. spectroscopy of bovine erythrocyte (Cass *et al.*, 1977*a*) and yeast (Cass *et al.*, 1978) superoxide dismutases and have published a preliminary note on the human erythrocyte enzyme (Stokes *et al.*, 1973). The chemical and physical properties of the human superoxide dismutase have been summarized (Bannister *et al.*, 1977). The u.v. and visible spectra, the e.p.r. spectra and the enzyme activities are not significantly different from the bovine enzyme. A partial amino acid sequence has been

determined (Barra *et al.*, 1978): of the 100 amino acid positions identified, nearly 90% are homologous to the bovine enzyme. More important is the fact that four out of six liganded histidines are conserved; the positions of the other two in the sequence have not been determined. We have carried out comparative studies of the two superoxide dismutases which may be of importance in relation to the proposed clinical use of the bovine enzyme (Michelson *et al.*, 1977; Huber & Saifer, 1977). In the present work ^1H n.m.r. spectroscopy has been used to investigate how far the sequence homology is reflected in structural similarities between the human and the bovine enzymes.

Experimental

Human superoxide dismutase was prepared by the methods previously described (Bannister *et al.*, 1977). The enzyme used in these studies corresponds to superoxide dismutase I. The apoprotein was prepared by dialysis (Weser & Hartmann, 1971; Fee, 1973) against 10 mM-EDTA at pH 3.8 for 3–4 days; any EDTA bound to the protein was removed by further dialysis against NaClO_4 . Solutions for n.m.r. experiments were prepared by dissolving the enzyme in $^2\text{H}_2\text{O}$. The solutions were left at room temperature for several hours and then freeze-dried; this procedure was repeated three times in order to exchange labile protons for deuterons.

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Immediately before the n.m.r. spectra were measured, samples were dissolved in $^2\text{H}_2\text{O}$ solutions of 1M-NaCl and 20mM-sodium phosphate, giving a final concentration of 100mg/ml (3mM). The Cu(I)/Zn(II) enzyme was prepared by reducing the Cu(II)/Zn form by addition of a few granules of sodium dithionite, so that the colour of the protein was just bleached. $^2\text{H}_2\text{O}$ (99.8% ^2H), ^2HCl and NaO^2H were purchased from Merck, Sharpe and Dohme (Montreal, Que., Canada) and CIBA (Basel, Switzerland) respectively. All other reagents were analytical grade, and the water was double distilled.

^1H n.m.r. spectra were recorded at 270MHz by using a modified Bruker HFX-90 console 6.4T superconducting magnet supplied by Oxford Instruments Ltd. (Oxford, U.K.) and a Bruker BNC 12 computer. Free induction decays were accumulated after a 70° pulse by using quadrature-phase detection, with a pulse-to-pulse time of 0.6s. The peak due to HO^2H was routinely suppressed by using a long-gated pulse before the measuring pulse (Campbell *et al.*, 1977). The estimation of exact peak intensities was made from spectra accumulated after a 90° pulse, with a much longer pulse-to-pulse time of 5s. Convolution difference spectra were calculated (Farrar & Becker, 1971) in order to increase the resolution of overlapping resonances by the method described by Campbell *et al.* (1973). Spin-echo spectra and spin-echo double-resonance spectra were obtained by $90-\tau-180-\tau-(\text{collect})$ pulse sequence as previously described (Campbell *et al.*,

1975a,b). For all spectra the deuterium resonance of the solvent $^2\text{H}_2\text{O}$ was used as an internal field-frequency lock and all chemical shifts are quoted in p.p.m. downfield of 2,2-dimethyl-2-silapentane-5-sulphonate used as an internal standard.

During pH titrations, the pH values were adjusted by addition of 500mM- ^2HCl and - NaO^2H solutions. The pH values of the protein solutions were measured, in the n.m.r. tube, with a Pye Ingold micro-electrode and Radiometer pH-meter 26, and pH values are quoted as direct meter readings, pH^* , uncorrected for the deuterium isotope effect.

Results

General features of the spectra of Cu(II), Cu(I) and human apo-(superoxide dismutase)

The convolution difference 270MHz ^1H n.m.r. spectra of the Cu(II)/Zn(II), Cu(I)/Zn(II) and apo-forms of human superoxide dismutase are shown in Figs. 1(a), 1(b) and 1(c) respectively. The spectra can be considered in two parts. The downfield region contains resonances from unexchanged NH protons, four phenylalanine, eight histidine and one tryptophan residues (for each 16000-dalton sub-unit). The upfield region is due to aliphatic methyl, methylene and methine groups. There is a total absence of ring-current-shifted resonances higher than 0.0p.p.m.

Fig. 1. ^1H n.m.r. convolution difference spectra of human (a) Cu(II)/Zn(II) , (b) Cu(I)/Zn(II) and (c) apo-(superoxide dismutase)

(a) The enzyme (3.3 mM) was in 20 mM-sodium phosphate/1 M-NaCl, $\text{pH}^* 5.87$ at $T = 313\text{ K}$; (b) the enzyme (3.3 mM) was in 20 mM-sodium phosphate/1 M-NaCl, $\text{pH}^* 5.79$ at $T = 313\text{ K}$; (c) the enzyme (3.0 mM) was in 20 mM-sodium phosphate/1 M-NaCl, $\text{pH}^* 5.77$ at $T = 313\text{ K}$. Abbreviation: TSP, 2,2-dimethyl-2-silapentane-5-sulphonate.

Fig. 2. ^1H n.m.r. convolution difference spectrum of the aromatic region of human apo-(superoxide dismutase). The enzyme (3 mM) was in 20 mM-sodium phosphate/1 M-NaCl, $\text{pH}^* 6.24$ at $T = 313\text{ K}$.

Spectra of the apoenzyme

The convolution difference spectrum of the aromatic region of the human apo-(superoxide dismutase) is shown in Fig. 2. Upon changing the pH^* of the solution, 12 resonances titrate (Fig. 3) with characteristics expected of resonances of C-2 and C-4 protons of the imidazole ring of the histidine residue (Markley, 1975). The correlation of resonances at high and low pH^* , especially *a*, *c*, *d*, and *e*, was made by taking into consideration the different associated line-widths. Since the resonances of C-2 protons usually occur at lower fields than those of the C-4 protons and the dependence of their shifts on pH^* is much more marked for the C-2 protons than in the C-4 protons, it is possible to use these criteria to divide the resonances into two groups. Resonances *a* and *c*–*h* titrate with a span of 0.55–1.2 p.p.m. and are assigned to C-2 protons and resonances *i*–*l* titrate with a span of 0.3–0.5 p.p.m. and are assigned to the C-4 protons. The curves shown in Fig. 3, except for that of resonance *a*, do not represent the pH^* titration of isolated groups, because they cannot be fitted to a normal Henderson–Hasselbach titration curve. Similar observations have been reported for bovine apo-(superoxide dismutase) and yeast apo-(superoxide dismutase) and have been interpreted as reflecting the mutual interaction of the histidine groups in the

Fig. 3. Titration curves of the C-2 and C-4 protons of the histidine residues of human apo-(superoxide dismutase).

metal-binding sites. The same explanation would imply that in the case of the human superoxide dismutases, six out of the eight histidine residues are located in the metal-binding sites and thus are probably metal ligands.

Resonance *b* exhibits a quite distinctive pH* behaviour. The chemical shift of the resonance is constant at pH* < 7.5, but at pH* > 7.5 moves upfield, broadens and eventually disappears. A ready $^1\text{H}/^2\text{H}$ exchange with the solvent above pH* 7.5 would explain the gradual disappearance of this resonance. The rate of exchange is similar to that reported (Cass *et al.*, 1977b) for a resonance in the spectrum of bovine apo-(superoxide dismutase). Such exchanges have been observed for a number of imidazole-containing compounds and also for a number of proteins including bovine superoxide dismutase. Because the rate of $^1\text{H}/^2\text{H}$ exchange for C-2 protons is faster than that of C-4 protons, resonance *b* is assigned to the C-2 proton of a histidine residue. The investigation (Cass *et al.*, 1977b) of the bovine erythrocyte enzyme by ^1H n.m.r. and ^3H exchange showed that an analogous histidine residue associated with the same anomalous behaviour is histidine-41. This residue must therefore be conserved in the human enzyme; it is absent from yeast superoxide dismutase.

Resonance *a* moves upfield as the pH* increases, and it follows a normal titration curve of a single ionizable group having a pK_a^* at 6.60. There is a C-2 proton resonance in the ^1H n.m.r. spectrum of

bovine apo-(superoxide dismutase) which titrates with a pK_a^* close to 6.70 and has been assigned in that protein to histidine-19 (Cass *et al.*, 1977a). Position 19 in the sequence of human superoxide dismutase is occupied by a Glx residue. Histidine-108 in human superoxide dismutase (replacing tyrosine-108 in the bovine enzyme) is probably associated with resonance *a*. If the structures of the bovine and human dismutases are alike, histidine-108 would be quite near the surface, consistent with the observed pK_a^* and the titration behaviour.

Cu(I)/Zn(II) enzyme

The convolution difference spectrum of the aromatic region of Cu(I)/Zn(II) human superoxide dismutase is shown in Fig. 4. Peaks 1–8 are assigned to C-2 protons and 9–12 to C-4 protons of the eight histidine residues. In contrast with the spectrum of the apoenzyme only three peaks are observed to titrate over the pH* range studied (pH* 5.00–9.15) (Fig. 5). Resonances 2–5, 7 and 8 show no pH*-dependence, suggesting that the histidine residues associated with these resonances in Cu(I)/Zn(II) act as ligands to the two metal ions. Peaks 6 and 12 in Fig. 4 are assigned to C-2 and C-4 protons respectively on the basis of their resonance positions as the span of their pH*-dependent shifts. The pK_a^* values for both resonances are close to 6.60, indicating that they arise from the C-2 proton and C-4 proton of the same histidine residue. Furthermore the pH*-titration curves of these resonances and the titration

Fig. 4. ^1H n.m.r. convolution difference spectrum of the aromatic region of human Cu(I)/Zn(II) superoxide dismutase. The enzyme was in 20 mM-sodium phosphate/1 M-NaCl, pH* 7.09, at $T = 313\text{ K}$.

Fig. 5. Titration curves of some of the resonances in the aromatic and the aliphatic regions of human Cu(I)/Zn(II) superoxide dismutase

curves of resonances *a* and *l* in the spectra of the apoenzyme are superimposable. It is therefore highly probable that resonances *a*, *l*, 6 and 12 arise from the same residue, histidine-108.

The rate of $^1\text{H}/^2\text{H}$ exchange of the residue which gives rise to resonance 1 is faster than the other C-2 proton resonances, but slower than that of resonance *b* in the spectrum of the apoenzyme, measured at the same pH^* and temperature. Nevertheless, resonances 1 and *b* are assigned to the same residue, histidine-41, on the basis of the fact that they are the only resonances that exchange at $\text{pH}^* < 8$.

Another resonance, T, is observed in the aromatic region. No such resonance is observed in the corresponding bovine superoxide dismutase spectrum. It may arise from tryptophan-30, which replaces a

threonine residue in the bovine sequence. Further work is required to confirm this tentative assignment.

In the aliphatic region the most striking difference between spectra of the bovine and human superoxide dismutase is the observation in the spectra of the latter of a resonance, denoted by S, which is a singlet, as concluded from an analysis of the spin-echo spectrum. At low pH^* it has a chemical shift of 1.97 p.p.m., and moves upfield gradually as the pH^* increases, as shown in Fig. 5. The curve can be fitted to a normal Henderson-Hasselbach titration curve. A single pK_a^* at 6.7 is obtained. It is unlikely to be due to the *N*-terminal *N*-acetyl group, which is probably associated with another resonance with a pH-independent chemical shift at 2.06 p.p.m. (Cass *et al.*, 1977a). Human superoxide dismutase contains a reactive cysteine residue, 109, forming (Briggs & Fee, 1978a,b) a trisulphide with glutathione. The spectrum of the sample of human superoxide dismutase used in these studies also shows an absorption at 325 nm characteristic (Briggs & Fee, 1978a) of this type of adduct. It is possible that S is associated with proton(s) in the group attached to cysteine-109, the chemical shift being influenced by the nearby histidine-108.

Cu(II)/Zn(II) enzyme

The convolution difference spectrum of the aromatic region of the oxidized holoenzyme is shown in Fig. 6. Owing to the effect of the paramagnetic Cu(II) ion on the relaxation times of neighbouring protons, there is a dramatic decrease in signal height (reflecting the increased line-width) in all but two of the histidine C-2 resonances, consistent with their role as metal ligands. Resonance 1, which has been assigned to histidine-41, is less (Richardson *et al.*, 1975a) than 1.2 nm away from the copper, and consequently its signal height is greatly reduced.

Resonance 6, which has been assigned to histidine-108, is not significantly affected by the paramagnetic Cu(II) ion. This is consistent with the fact that histidine-108 is near the surface and far away from the copper site. Resonance 6 and the corresponding resonance 12 titrate normally, with a pK_a^* value of 6.4, sufficiently similar to those of the corresponding resonances in the spectra of the apoenzyme and Cu(I)/Zn(II) protein to suggest that the change in the oxidation state of the copper only slightly affects this residue, histidine-108.

Resonance N, whose chemical shift is not dependent on pH^* , and resonance S, whose is, are observed in the aliphatic region of the spectra. The titration curve of resonance S is similar to that in Fig. 5 with a pK_a^* of 6.4. The near-identical pK_a^* of resonance S and resonance 6 in the oxidized holoenzyme is consistent with the assignment of reson-

Fig. 6. ^1H n.m.r. convolution difference spectra of the aromatic region of human Cu(II)/Zn(II) superoxide dismutase. The enzyme was in 20 mM-sodium phosphate/1 M-NaCl, pH* 6.97 at $T = 313\text{ K}$.

ance S to the adduct of cysteine-109, since, if the structures of the bovine and human enzymes are similar, this residue will also be far from the Cu(II) ion.

Discussion

The ^1H n.m.r. spectra of human apo-, Cu(I)/Zn(II) and Cu(II)/Zn(II) superoxide dismutase are sufficiently similar to those of the corresponding forms of bovine superoxide dismutase to suggest that the structures in solution are closely related, which is consistent with the significant homology in the amino acid sequences. All the assigned residues in bovine, human and yeast superoxide dismutases are given in Table 1. Of the six histidine resonances in the spectra of human superoxide dismutase that we have assigned as ligand histidine, the chemical shifts of five are remarkably similar to those assigned in the spectra of bovine superoxide dismutase. Such is the similarity that we predict that all six histidine ligands are conserved and that positions 43 and 78 in the human superoxide dismutase amino acid sequence are occupied by histidine residues. The behaviour of the resonances assigned to histidine-41 in human superoxide dismutase, the anomalous $^1\text{H}/^2\text{H}$ exchange behaviour and the effect of paramagnetic Cu(II) ion is paralleled by that assigned to histidine-41 in the bovine protein. This suggests that the environment of this residue, which must be somewhat special to account for the anomalous exchange behaviour, must be strongly conserved. It is possible therefore that the whole β -barrel structure is retained in these two proteins. Immunological cross-reactivity as well as hybridization studies (Huber & Saifer, 1977) also suggest that there is little surface alteration and moderate compatibility of the intersubunit interfaces.

Table 1. Comparison of chemical shifts of assigned residues in Cu(I)/Zn(II) superoxide dismutase from bovine and human erythrocytes and yeast

The numbering of resonances refers to Fig. 1. The results for the bovine enzyme are taken from Cass *et al.* (1977a), and the results for the yeast enzyme are taken from Cass *et al.* (1978).

Resonance	Chemical shift (p.p.m.)		
	Bovine	Human	Yeast
(1) Histidine-41 C-2H	8.62	8.63	—
(6) Histidine-19/108 C-2H	8.10	8.05	—
(12) Histidine-19/108 C-4H	6.75	6.67	—
(2) Histidine C-2H	8.58	8.53	8.54
(3) Histidine C-2H	8.47	8.48	8.59
(4) Histidine C-2H	8.33	8.35	8.30
(5) Histidine C-2H	—	8.15	—
(7) Histidine C-2H	7.72	7.76	7.70
(8) Histidine C-2H	6.50	6.48	6.50
Tyrosine	7.01	—	7.05
Tyrosine	6.67	—	6.77
N N-Terminal N-acetyl	2.08	2.09	—
S	—	1.79	—
T Tryptophan-30 C-2H	—	7.99	—

What surface alteration there is may be associated with the major difference between the human and bovine erythrocyte superoxide dismutases, i.e. the presence of a modified cysteine residue in the former. If our assignment of resonance S is correct, it is possible that the glutathione moiety attached to cysteine-109 (Briggs & Fee, 1978a,b) interacts with both the neighbouring histidine and aspartate residues.

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