Investigation of the Structure of Bovine Erythrocyte Superoxide Dismutase by ^{1}H Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: The 270-MHz ^{1}H nuclear magnetic resonance spectra of the apo, copper(I)-zinc(II), and copper(II)-zinc(II) forms of bovine erythrocyte superoxide dismutase (EC 1.15.1.1) are reported, and assignments of resonances to ten amino acid residues are proposed. The data require that at least four and probably six histidine residues serve as ligands to the metals in each subunit of the enzyme, consistent with x-ray diffraction results. The remaining assigned resonances are associated with His-19, His-41, Tyr-108, and the N-terminal *N*-acetyl group. The imidazole C(2)H of His-41 exchanges readily at pH* >8. The structural implications of the effect of the paramagnetic Cu(II) in the holoenzyme on the proton relaxation times are in reasonable accord with the data from x-ray diffraction studies.

Usually the comparison of structures in the crystalline and solution phases presents few problems when the molecular weight of the compound of interest is low. With proteins, however, only a few attempts have been made to effect such comparisons and in most instances only a limited comparison has been possible. Recently we have attempted to deduce many features of the structures of some copper proteins in solution (Hill et al., 1976). In doing so, it became obvious that we needed to test our basic assumptions on a copper protein whose structure in the crystalline phase was known. Ironically the first, and to date the only, copper protein whose structure (Richardson et al., 1975a,b) in the crystalline phase is known is bovine erythrocyte superoxide dismutase, a protein we had cursorily investigated (Stokes et al., 1973, 1974). Since then, apart from the structure determination, there have been a number of advances in the understanding of the chemistry of this metalloenzyme (Fridovich, 1975). We therefore considered it timely to investigate the ^{1}H NMR[1] spectroscopy of superoxide dismutase with the advantage of being able to employ a number of recent advances in NMR techniques and direct our work not only to the problem of structure determination in solution, in which context one may consider superoxide dismutase a "model" copper protein, but also toward elucidation of the mechanism of the enzymatic reaction. This paper describes some ^{1}H NMR experiments necessary before proceeding far along the road toward the attainment of these goals.

Materials and Methods

Bovine superoxide dismutase was prepared by the methods previously described (Bannister et al., 1971). Apoprotein was prepared by dialysis against 10 mM EDTA, pH 3.8 (Weser and Hartmann, 1971), and any EDTA bound to the protein was removed by dialysis against sodium perchlorate (Fee, 1973). Protein solutions (approximately 150 mg/mL) for the NMR experiments were prepared in 0.02 M sodium phosphate buffer containing 1 M sodium chloride in 99.8% D_2O. The protein solutions were allowed to equilibrate with D_2O solvent at room temperature, over a period of hours, in order to facilitate deuteration of exchangeable protons, freeze-dried, then taken up in fresh D_2O prior to NMR experiments.

A Pye Ingold microelectrode attached to a Radiometer pH meter 26 was used for pH measurement. NaOD or DCl solutions were used for pH adjustments and pH and pK values quoted (as pH* and pK*) are uncorrected for the deuterium isotope effect. When required, the holoenzyme was reduced with sodium dithionite.

^{1}H NMR spectra were obtained at 270 MHz using a modified Bruker HFX-90 console, 6.4 T superconducting magnet (Oxford Instruments Ltd.) and Nicolet 1085 computer, of the Oxford Enzyme Group. Free induction decays were collected in 4096 data points at a sampling frequency of 8000 s^{-1} and with a pulse-to-pulse separation of 0.6 s. Convolution difference spectra were calculated by the usual method (Campbell et al., 1973). The conditions used for spin-echo spectra and spin-echo double resonance experiments were much as described in the literature (McLaughlin et al., 1973; Campbell and Dobson, 1975; Campbell et al., 1975b). Transverse relaxation times of resonances in the copper(I)-zinc(II) superoxide dismutase spectra were obtained using the pulse sequence: $90_x-\tau-(180_y-2\tau)_n-180_y-\tau$[collect], where τ is a time (chosen as 1 ms), n is a variable integer, and the subscripts x and y signify a 90° phase shift between applied 90 and 180° pulses. The total time between the initial pulse and collection of the free induction decay was varied between 10 and 100 ms and T_2 values were gained from the usual (Farrar and Becker, 1971) semilogarithmic plots. For all spectra the deuterium resonance of the D_2O solvent was used as an internal field-frequency lock. Chemical-shift values are quoted as parts per million (ppm) downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard.

Results and Discussion

General Features of the Spectra. The conventional 270-MHz ^{1}H NMR spectra of the copper(I)-zinc(II) and copper(II)-zinc(II) forms of bovine superoxide dismutase are shown in Figures 1A and 1B, respectively. The region of major

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[1] Abbreviations used: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EPR, electron paramagnetic resonance.

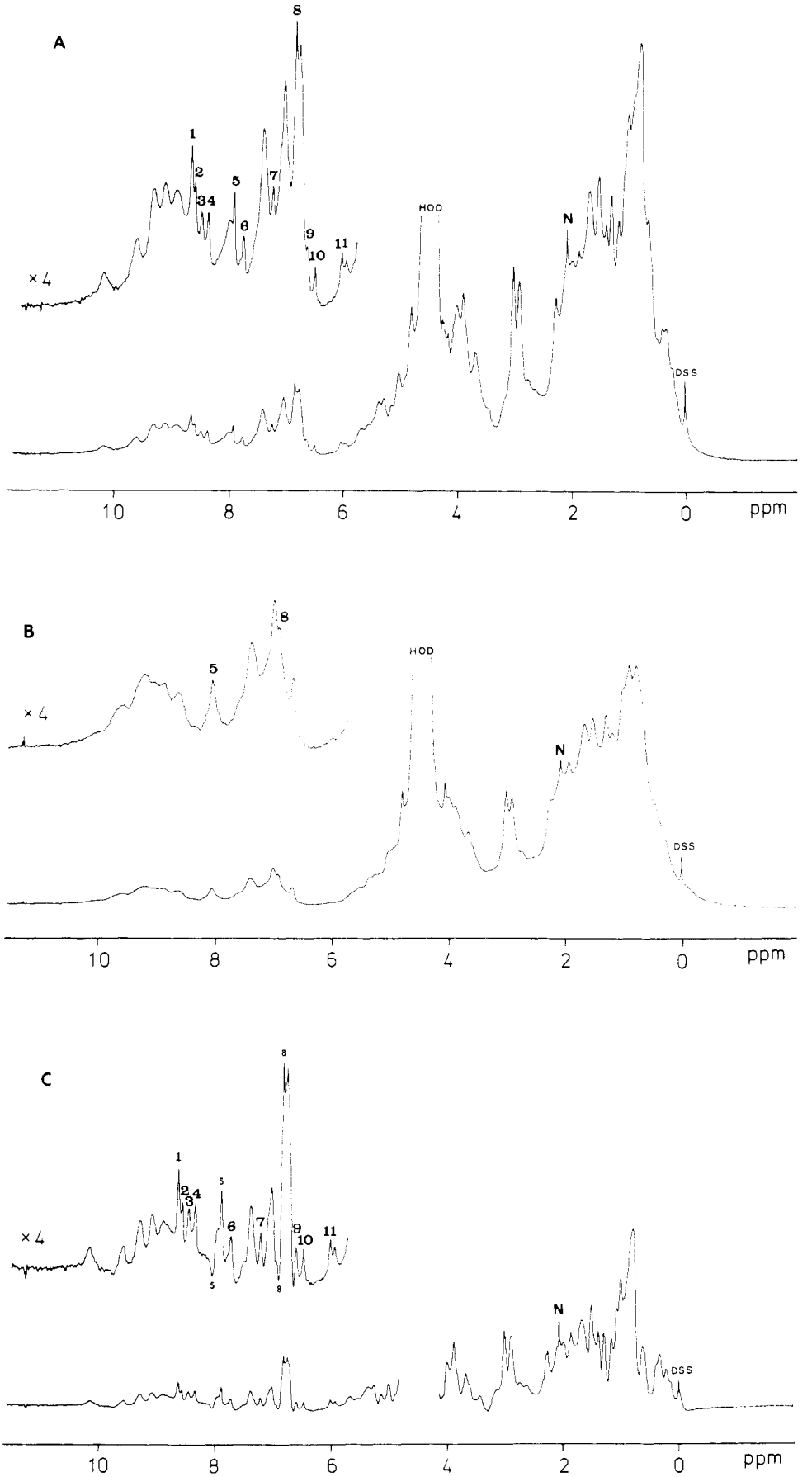

FIGURE 1: The conventional 270-MHz ^{1}H NMR spectra of bovine erythrocyte superoxide dismutase. Copper(I)-zinc(II) enzyme at 50 °C and pH* 7.05 (A); copper(II)-zinc(II) enzyme at 50 °C and pH* 6.9 (B). The difference spectrum C is obtained by subtracting spectrum B from spectrum A. The appearance of peaks 5 and 8 in the difference spectrum is due to the slight pH* difference between copper(I)-zinc(II) and copper(II)-zinc(II) samples.

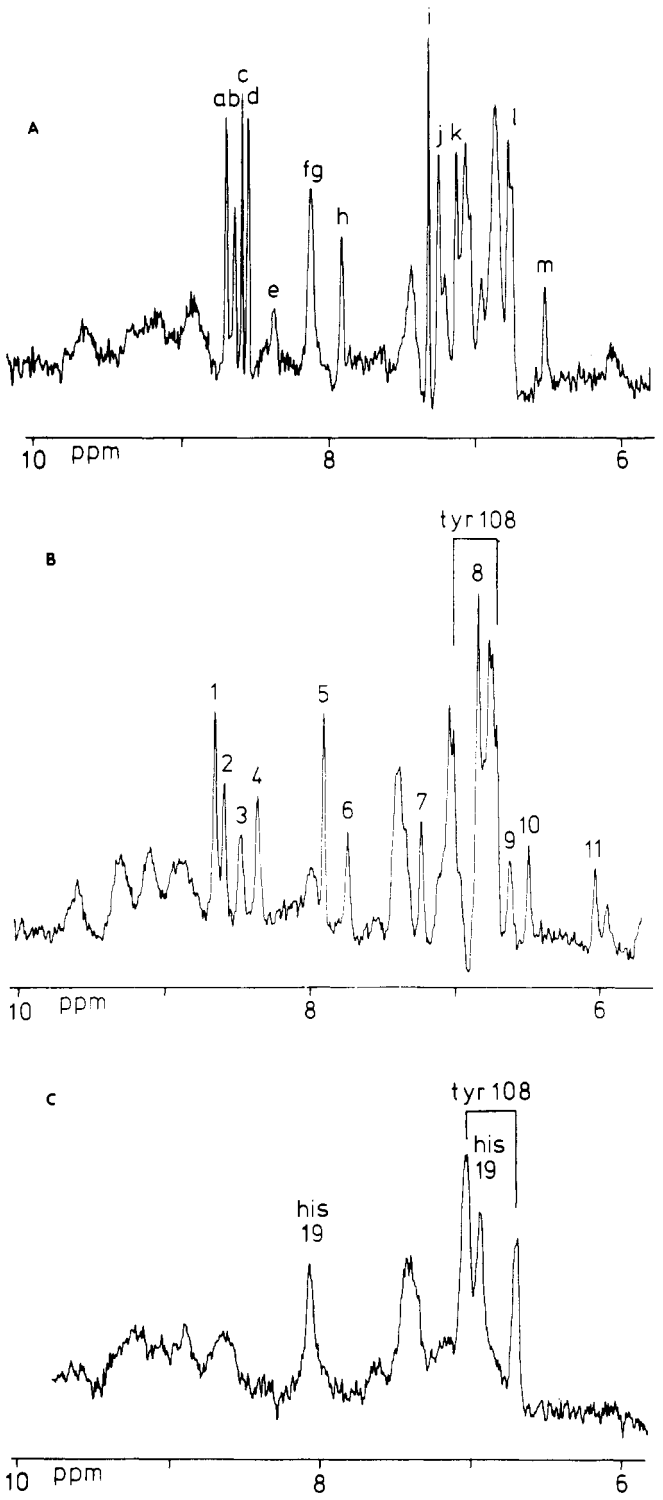

FIGURE 2: Convolution difference spectra of the low-field region of various forms of bovine erythrocyte superoxide dismutase. Apo (A), copper(I)-zinc(II) (B), and copper(II)-zinc(II) (C).

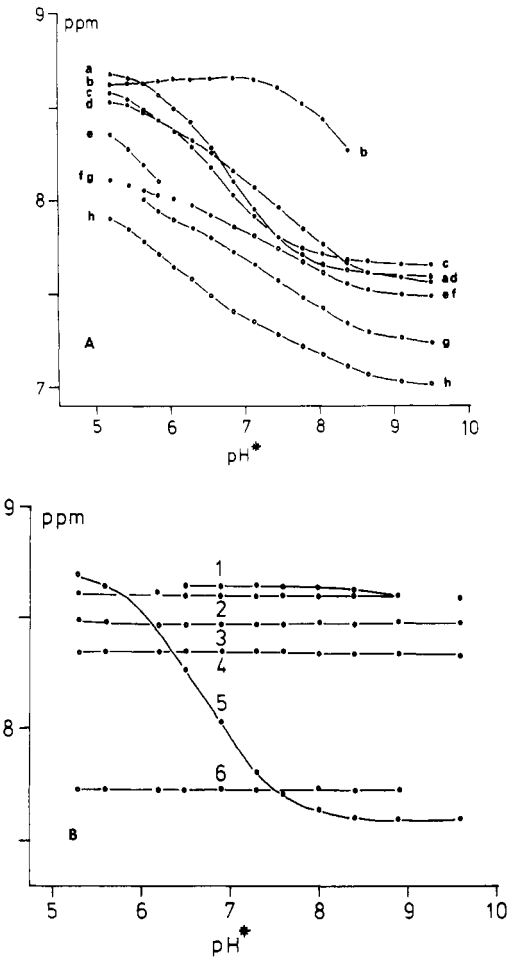

FIGURE 3: The dependence of chemical shift on pH* for the C(2) protons of various histidine residues in apo (A) and copper(I)-zinc(II) (B) bovine erythrocyte superoxide dismutase. The labeling of the curves corresponds to that used in Figures 1 and 2. Both sets of data were obtained at 40 °C.

spectral intensity, upfield of the solvent HOD peak, is due to ubiquitous aliphatic methyl, methylene, and methine groups, whereas the low-field region (chemical shift > 6 ppm) contains resonances from aromatic residues and unexchanged NH protons. The latter are expected (Campbell et al., 1975a) to be distributed over a wide chemical-shift range but are most clearly identified in the present data as a rather broad envelope between 8 and 10 ppm. In the spectrum of the copper(I)-zinc(II) form of bovine superoxide dismutase (Figure 1A), the remaining low-field resonances derive from four phenylalanine, one tyrosine, and eight histidine residues (for each 15 600-dalton subunit). Considerable resolution enhancement is achieved in the convolution difference spectra of this region of apo-, copper(I)-zinc(II), and copper(II)-zinc(II) proteins, shown in Figures 2A, B, and C, respectively. In the following, assignments of some of the observed resonances will be described and structural information will be deduced from comparisons between the spectra of, firstly, apo- and copper(I)-zinc(II) proteins and later, copper(I)-zinc(II) and copper(II)-zinc(II) proteins.

Spectra of Histidines in Apo- and Cu(I)-Zn(II) Proteins. Generally histidine imidazole C*H* protons appear as relatively sharp resonances with the C(4) proton occurring in the region of major aromatic intensity (approximately 6.5 to 7.5 ppm) and C(2) protons distinctively to lower field (approximately 7.5 to 9 ppm). Where the imidazole nitrogen is freely amenable to deprotonation, an increase in pH results in a characteristic (Markley, 1975a) continuous decrease in C(2)H and C(4)H chemical shifts. Application of these criteria has led to the assignment of resonances to all eight histidine residues (per subunit) in the spectra of apo-superoxide dismutase. In Figure 2A, resonances a to h are due to C(2) protons and resonances i to m correspond to C(4) protons. The pH dependence of the eight C(2)H chemical shifts is shown in Figure 3A. By contrast, resonances due to six histidine residues have been as-

signed[2] in the copper(I)-zinc(II) spectra. In Figures 1A and 2B, peaks 1 to 6 are assigned to C(2)H and peaks 7 to 11 to C(4)H, and only one imidazole side chain is observed to titrate over the pH range studied (Figure 3B). This very marked difference between the histidine titration characteristics of the apo- and the copper(I)-zinc(II) protein immediately implicates a number of histidine residues in the ligation to copper and zinc centers in the holoenzyme. Resonances 5 and 8 in Figures 1A and 2B belong to the C(2) and C(4) protons, respectively, of the only freely titrating histidine residue, in the copper(I)-zinc(II) enzyme, both peaks showing a pK* of 6.7. This residue is clearly a nonligand. Its ionization characteristics remain largely unchanged on removal of the metals, and indeed the titration curves of resonances 5 and 8 are nearly superimposable on those of resonances a and k from the spectrum of the apoenzyme.

Exchange of a Histidine C(2)H. Figure 3A shows that, of the eight histidine residues of apo-superoxide dismutase, the residue associated with resonance b exhibits a quite distinctive pH behavior. The C(2)H chemical shift remains unaffected until pH* 7.5, whereupon the resonance moves upfield and broadens, eventually beyond detection. In separate experiments, incubation of superoxide dismutase at pH* 8.9 and 40 °C for 4 h in D_2O resulted in the complete and selective loss of resonance b (apoprotein) or resonance 1 (copper(I)-zinc protein). In neither case was intensity recovered by lowering the pH, but in both cases prolonged (approximately 12 h) incubation of the resultant proteins under the same conditions but in H_2O rather than D_2O, yielded a return of peak intensity. We conclude that the experimental observations relate to the (reversible) deuteration of imidazole C(2)H in D_2O solvent at high pH. Such exchanges have been observed for a number of imidazole derivatives (Olafson et al., 1964; Harris and Randall 1965; Vaughan et al., 1970) and more recently for histidine residues in a number of proteins (Markley, 1973a,b, 1975a,b; Matsuo et al., 1972; Ohe et al., 1974). The similar facility with which the residues which give rise to resonances b and 1 undergo C(2)H deuterium exchange suggests that they are one and the same residue. This is supported, in the absence of an unfortunate coincidence, by the identical (8.65 ppm) chemical shifts. Furthermore these two observations are consistent with the proposal that this residue is not a ligand to either the copper or the zinc since it would be expected that both the exchange and the chemical shift would be much affected by ligation to a metal ion. The absence of evidence of histidine–histidine interactions (vide infra) in the pH-titration curve of resonance b provides further support for this proposal.

Metal Ligands. A marked change in the pH-titration characteristics of a histidine residue is expected on ligation of imidazole nitrogen to a metal. We have already considered the pH behavior of resonances a and b in the spectrum of the apoprotein and their analogues in that of the reduced protein, 5 and 1, respectively. It is notable that, whereas the remaining six histidines in the apoprotein all titrate (corresponding to resonances c to h), the additional histidine resonances in the spectrum of the copper(I)-zinc(II) protein do not titrate over the range, pH* 5.3 to 9.6, reflecting a significant change in the pK*. Four of the six are clearly identified in Figures 1A and 2B as resonances 2 (8.61 ppm), 3 (8.48 ppm), 4 (8.35 ppm), and 6 (7.73 ppm). It is therefore possible to conclude, *solely from the present study by NMR spectroscopy*, that there are at least four and probably six histidine ligands to the copper-zinc center in each subunit of bovine superoxide dismutase. Two histidines are nonligands. These conclusions are entirely consistent with recent x-ray diffraction results (Richardson et al., 1975a,b) from which three histidines were found to be bound to copper (His-44, -46, and -118), two are bound to zinc (His-69 and -78), one may act as a bridging ligand between copper and zinc (His-61), and the remaining two (His-19 and -41) are nonligands.

Knowledge of the metal ligation provides a plausible explanation of some of the unusual titration curves in Figure 3A. Removal of both copper and zinc leaves a number of histidines which may be in sufficient proximity to allow complex His–His interactions. In this case titration of one or more groups could affect the chemical shift and/or the state of ionization of the observed histidine. Treatment of such effects has been given (Schrager et al., 1972; Markley and Finkenstadt, 1975) for the case of two interacting components. Finally, apart from the specific comparison of apo- and copper(I)-zinc(II) proteins above, a comparison of the overall spectral pattern of these systems (at the same temperature and pH) reveals that there are a number of distinct differences. While this necessarily implies that removal of metals is associated with structural changes, it is difficult at this stage to gauge the nature or extent of the effect. It has been concluded from studies by circular dichroism (Wood et al., 1971) that there is little change in the regular secondary structure of apo- and holoenzymes, though others (Fee and Phillips, 1975) have implied larger changes.

Additional Assignments in the Spectrum of the Copper(I)-Zinc(II) Enzyme. For further assignments in the ^1H NMR, spin-echo methods (McLaughlin et al., 1973; Campbell and Dobson, 1975; Campbell et al., 1975b) have been employed. The spectrum of copper(I)-zinc(II) superoxide dismutase shown in Figure 4 results from the pulse sequence 90-τ-180-τ[collect] with the time τ = 50 ms. The long period between the initial 90° pulse and the collection of the free induction decay (100 ms) allows for considerable dephasing of the magnetization in the xy plane and hence only the sharpest of lines (long T_2) are observed, leading to a marked spectral simplification. Further, for multiplets characterized by a coupling constant J (Hz), choice of the time 2τ in the vicinity of n/J (n as an integer ≥ 1) results in doublets appearing in the spectrum 180° out of phase (i.e., inverted) with respect to singlets and triplets. Thus, in addition to the histidine singlets shown in Figure 4, two doublets at 6.67 and 7.01 ppm are also clearly observed. Decoupling experiments (see Campbell and Dobson, 1975) reveal that both doublets derive from the same amino acid residue, and, since only the ring protons of a tyrosine residue can give rise to two related doublets in this region, these resonances can be assigned to the ortho and meta protons of Tyr-108. In Figure 1A a sharp resonance, denoted N, is well defined at 2.08 ppm. In the spin-echo spectrum, this feature is also clearly observed and shows phase characteristics consistent with a singlet. There are two possible assignments for this resonance: the methyl group of Met-115, or the methyl group of the N-terminal (Evans et al., 1974) N-acetyl group. The results derived from the study of the effect of the paramagnetic copper(II) ion allow, when taken in conjunction with the structure determined by x-ray diffraction, a distinction to be made between these two possible assignments.

Paramagnetic Broadening in the Spectrum of the Copper(II)-Zinc(II) Enzyme. Large magnetic fields deriving

[2] The chemical shift of resonance 7 is not inconsistent with assignment to a histidine C(2) proton. This interpretation would imply the observation of resonances due to seven histidines in the spectrum of the copper(I)-zinc(II) enzyme. We prefer the more conservative assignment to a C(4) proton. The conclusions drawn later in the text are not affected.

from the unpaired electron spin in the oxidized copper(II)-zinc(II) form of the enzyme provide an efficient mechanism for relaxation of the nuclear spins of proton groups in the vicinity of the copper(II) center. The Solomon treatment of dipolar paramagnetic relaxation (Solomon, 1955) yields the expression:[3]

$$\frac{1}{T_{2M}} = Kr^{-6}\left(4\tau_C + \frac{3\tau_C}{1+\omega_I^2\tau_C^2} + \frac{13\tau_C}{1+\omega_S^2\tau_C}\right)$$

where K is a collection of constants, r is the proton-paramagnetic center distance, ω_I and ω_S are ^1H NMR and EPR frequencies respectively, and τ_C is a correlation time characteristic of the electron-nuclear dipolar interaction.

A comparison of the spectra of the diamagnetic copper(I)-zinc(II) and paramagnetic copper(II)-zinc(II) superoxide dismutases (Figures 1A and 1B) shows that the latter displays increased intensity in aliphatic, aromatic, and unexchanged NH regions. This is particularly clear in the difference spectrum given in Figure 1C and derives from the paramagnetic contribution to the line widths. While the range of effect of the copper(II) center is critically dependent on the value of τ_C, Figure 1C shows that there is some specificity, and in the absence of a knowledge of τ_C this forms the basis of a qualitative probe of copper-proton distances. Indeed the presence of specificity is expected from quite simple considerations. For strongly bound copper, τ_C is given by

$$1/\tau_C = 1/\tau_R + 1/\tau_e$$

where τ_R is a correlation time characteristic of molecular tumbling and τ_e is the electron spin relaxation time. The maximum value of τ_C possible is τ_R and this is calculated from the Stokes-Einstein equation[4] as approximately 8×10^{-9} s for superoxide dismutase under the present conditions. The height of a peak appearing in the difference spectrum is a function of both the paramagnetic broadening and the initial line width of the resonance in the spectrum of the diamagnetic protein (Dwek et al., 1975). The transverse relaxation times of resonance 1 to 6 in the spectrum of copper(I)-zinc(II) superoxide dismutase (40 °C, pH* 6.6) have been measured as 46, 38, 21, 31, 64, and 30 ms, respectively. These are among the sharpest lines ($\nu_{1/2} = 1/(\pi T_2)$) in the spectrum. For a resonance of line width at half-height of 10 Hz, and for a value of $\tau_C = \tau_R \approx 8 \times 10^{-9}$ s, the difference spectrum peak height will be $\geq$75% of that in the diamagnetic protein spectrum for a Cu-^{1}H distance $\leq$13 Å. Since a value of $\tau_C < \tau_R$ results in a smaller range of effect of the paramagnetic center, and a diamagnetic line width greater than 10 Hz results in a smaller fractional difference spectrum peak height, the above distance of 13 Å represents an approximate upper limit, within which a resonance may be broadened "beyond detection" by the copper(II) center in oxidized holo-superoxide dismutase. In the following, the structural implications of a comparison between copper(I)-zinc(II) and copper(II)-zinc(II) NMR spectra are considered in relation to average side chain coordinates derived[5] from x-ray diffraction results.

The C(2)H and C(4)H resonances of one histidine residue only are clearly distinguishable in the spectrum of copper(II)-zinc(II) superoxide dismutase. These titrate with pK* of 6.7 and therefore derive from the same residue as peaks 5 and 8 in the copper(I)-zinc(II) spectrum and peaks a and k in the apo-superoxide dismutase spectrum. The second nonligand histidine is not observed and consequently appears clearly in the difference spectrum (Figure 1C). The x-ray data indicate that the imidazole side chains of His-19 and -41 are 20 and 12 Å, respectively, from the copper atom. Thus, in Figures 1A and 2B, peaks 5 and 8 are assigned to His-19 and peak 1 to His-41. All histidine ligands are within 8 Å of the copper. As expected, resonances due to four of these (2, 3, 4, and 6 in Figures 1A and 2B) are broadened beyond detection in the spectra shown in Figures 1B and 2C and are clearly evident in the difference spectrum. The sharp resonance marked N in Figure 1A appears only slightly broadened in Figure 1B, thus providing a relatively weak contribution to the difference spectrum (Figure 1C). The average side chain coordinates of Met-115 place this residue 8.5 Å from the paramagnetic center. We therefore assign resonance N to the more distant methyl group of the N-acetyl group. The increased resolution of the histidine C(4)H resonance 9, in Figure 1C, results from the minor contribution of the adjacent high-field tyrosine doublet to the difference spectrum. The average (x-ray) copper-side chain distance for Tyr-108 is 18 Å, and in keeping with this the ortho and meta proton doublets are observed in the conventional and spin-echo NMR spectra of the copper(II)-zinc(II) enzyme. The remaining difference spectrum intensity between 6 and 8 ppm in Figure 1C derives from unexchanged NH and phenylalanine ring protons. The closest phenylalanine side chains are from Phe-43 and -62. These are approximately 9 Å from the paramagnetic center and are adjacent, in the protein primary sequence, to histidine residues which serve as ligands to the copper.

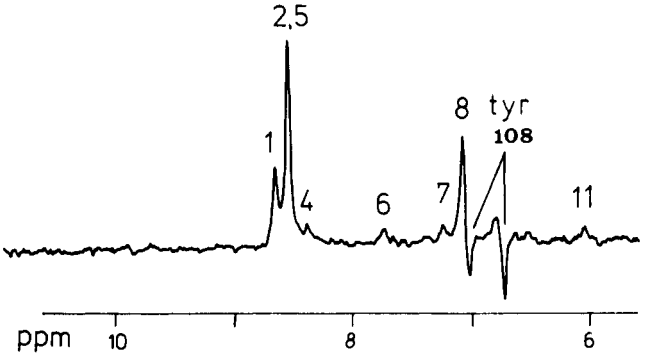

FIGURE 4: The spin-echo spectrum of copper(I)-zinc(II) bovine erythrocyte superoxide dismutase at 60 °C and pH* 5.6. The pulse sequence employed is of the form 90-τ-180-τ[collect] with the time τ set to 50 ms. The peak numbering scheme is as used in Figures 1A, 2B, and 3B.

It is of value to distinguish between those elements of the above analysis which can be derived solely from the application of NMR spectroscopy and those which are dependent on a prior knowledge of the structure as determined by x-ray diffraction studies. The conclusion that at least four and probably six histidine residues serve as ligands to the metal ions in the holoenzyme is required by the NMR data. The assignment of nonligand histidine resonances to specific residues, viz., His-19 and His-41, depends on a foreknowledge of the x-ray structure (see, however, footnote 6), as does the assignment of resonance

[3] Only terms for the dipolar relaxation mechanism are included and it is assumed that $\omega_S \gg \omega_I$. Contact interactions are expected to be restricted to ligand residues.

[4] $\tau_R = (M\bar{V}\eta)/RT$ where M = molecular weight (31 200), $\bar{V}$ = partial specific volume (0.72 cm^3 g^{-1}), and η is the viscosity, taken from values of 20% sucrose solutions. A spherical shape is assumed.

[5] Kindly supplied by Dr. D. C. Richardson.

[6] Since this manuscript was submitted, the assignment of resonance 1 to His-41 has been confirmed by tritiation of the C(2) proton and subsequent peptide mapping (A. E. G. Cass, H. A. O. Hill, B. E. Smith, J. V. Bannister, and W. H. Bannister, to be published).

N to the *N*-acetyl residue rather than Met-115. It is of particular interest that the relative copper–proton distances deduced from the effects of the paramagnetic copper(II) ion on the relaxation times are consistent with those emanating from the x-ray structure. This has the important consequence that, at the very least, qualitative values of metal–proton distances may be derived from the NMR spectra of copper proteins. It is intended that the assignments made in the present work, since they allow information to be gained on a variety of amino acid residues distributed throughout the protein, should serve as a basis for further studies of the structure and reactivity of bovine erythrocyte superoxide dismutase.

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