Nuclear Magnetic Resonance and Chemical Modification Studies of Bovine Erythrocyte Superoxide Dismutase: Evidence for Zinc-Promoted Organization of the Active Site Structure†

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ABSTRACT: Nuclear magnetic resonance (NMR) spectroscopy of the exchangeable protons, tentatively assigned as histidine resonances, of bovine erythrocyte superoxide dismutase in H_2O has been found to be a powerful method to study the active site of the enzyme. This technique has been employed in conjunction with chemical modification of the histidine residues using diethylpyrocarbonate (DEP) to show that zinc alone organizes the active site structure. All eight histidines per subunit of apoenzyme react with DEP. The accessibility of these residues to solvent is borne out by the broad, featureless NMR spectrum of the apoprotein. In the holoenzyme only His-19, which is exposed to solvent, can be modified with DEP. The reduced holoenzyme shows a well-resolved NMR spectrum compared with the oxidized form in which the lines are broadened by the paramagnetic copper ion. A spectrum very similar to that of the reduced enzyme is generated by addition of one zinc ion per subunit of apoprotein showing that zinc alone restores much of the native structure. This interpretation is supported by the fact that addition of up to 1 mol of zinc per subunit statistically reduces the number of histidine residues that can be modified by DEP until, at Zn:apoprotein ratios ≥ 1, only His-19 reacts. The NMR spectrum of the apo plus 2 Zn^{2+} protein has additional structure that is briefly discussed.

Bovine erythrocyte superoxide dismutase (SOD[1], molecular weight 31 200) has two identical subunits, each of which contains one copper(II) ion and one zinc(II) ion (Fridovich, 1975). X-ray structural (Richardson et al., 1975a,b) and sequence (Steinman et al., 1974) studies show the copper and zinc ions to be in close proximity, apparently bridged by the imidazole ring of His-61. The other amino acids that provide coordinating ligands have been identified as His-44, His-46, and His-118, which complete the approximately square planar coordination about copper, and His-69, His-78, and Asp-81, which complete an approximately tetrahedral environment for zinc. There are eight histidine residues per subunit. The two remaining ones are His-19, which is known from the x-ray studies to be accessible to solvent, and His-41, which is less accessible since it is buried in the β-barrel structure of the protein.

The zinc ion in bovine SOD is unique among those zinc metalloenzymes for which structural and mechanistic information is available. Zinc is found in a large number of metalloenzymes, either at a catalytic site where hydrolysis reactions occur or in a site removed from the catalytic center of the enzyme (Dunn, 1975; Chlebowski and Coleman, 1976). The copper ion in bovine SOD has been established by pulse radiolysis experiments to be the redox center responsible for the catalytic dismutation of superoxide ion (Klug-Roth et al., 1973; Fielden et al., 1974). The proximity of the zinc ion to this center is an unusual feature and suggests that zinc plays an important part in the functioning of the enzyme. At present, however, the role of the zinc ion has not been definitively established, although both structural (Rotilio et al., 1972; Forman and Fridovich, 1973) and specific functional (Hodgson and Fridovich, 1975; Lippard et al., 1977) roles have been suggested. Neither of these suggestions requires direct attachment of the substrate to zinc in the enzyme mechanism, in accord with the fact that, unlike the copper ion, the zinc ion is inaccessible to solvent (Fee and Ward, 1976).

The large number of histidine residues associated with the active site of bovine SOD facilitates study of its chemical and structural properties. In this report we describe NMR studies of the exchangeable protons and chemical modification of the histidine residues using diethylpyrocarbonate (DEP). The results help define the role of zinc in bovine SOD. They also demonstrate the potential of these physical and chemical probes to provide detailed information about the active site structure and, hopefully, the enzyme mechanism.

Materials and Methods

Bovine erythrocyte SOD was isolated and purified according to the method of McCord and Fridovich (1969). Material eluted from the final DEAE-52 cellulose column was dialyzed against deionized water, lyophilized, and stored as a powder at −20 °C. The electronic and EPR spectral properties of the enzyme matched those reported in the literature (Keele et al., 1971). Its activity was assayed according to a modification of the procedure of Misra and Fridovich (1972). The reaction was initiated by the addition of 50 μL of a 2.5 M sodium carbonate solution, pH 10.4, to 2.5 mL of a solution containing 0.3 mM epinephrine, 0.1 mM EDTA, and 1 mM HCl. The final pH was 10.2. The assay was completed as described (Misra and Fridovich, 1972). The specific activity of the enzyme ranged from 80 to 100% of the literature value. Most preparations

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[1] Abbreviations used are: SOD, superoxide dismutase; DEP, diethylpyrocarbonate; TSP, 3-(trimethylsilyl)propanesulfonic acid; EDTA, disodium salt of ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; AA, atomic absorption; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; CW, continuous wave; UV, ultraviolet.

showed two bands on polyacrylamide gel electrophoresis, run according to the method of Ornstein (1964).

Apo-SOD was prepared by overnight dialysis at room temperature against 1–10 mM EDTA in 50 mM sodium acetate at pH 3.8. Enzyme-bound EDTA was removed by exhaustive dialysis at 4 °C against the same buffer in which the EDTA was replaced by 100 mM sodium chloride or sodium perchlorate (Fridovich, 1973). Samples of apo-SOD prepared for NMR work were then dialyzed against deionized water and lyophilized. For chemical modification studies, the salt was removed by exhaustive dialysis against pH 3.7 acetate buffer at 4 °C and the resulting desalted apo-SOD solution (~30 μM) was stored under these conditions until further use. Metal analysis of this apo-SOD preparation by atomic absorption (AA) photometry (Perkin-Elmer Model 360) showed in one sample the presence of 0.07 equiv of zinc and 0.04 equiv of copper per subunit. Another preparation contained zinc below the limits of detection and 0.05 equiv of copper per subunit.

The concentrations of protein stock solutions used in the chemical modification studies were determined spectrophotometrically taking ϵ_{258} = 10 300 (McCord and Fridovich, 1969) for native SOD and ϵ_{258} = 2920 (Fee, 1973) for apo-SOD. Concentrations of apoenzyme less than 3.0 μM, however, were measured by the Lowry method using native bovine SOD as a calibrant. Two calibration curves were prepared, one of which was based on a weighed amount of lyophilized powder (mol wt = 31 200) and the other was established spectrophotometrically using ϵ_{258} = 10 300. These two calibration curves agreed quite well with one another at low protein concentrations but at about 2.6 μM the difference between them was 0.12 μM. The uncertainty introduced by this discrepancy into the results of the chemical modification experiments is at most ±0.3 ethoxyformylated histidines (vide infra). Enzyme concentrations in the NMR studies were determined by weighing out the lyophilized powder and taking the molecular weight to be 31 200 and 31 000 for the native and apoproteins, respectively.

All buffers were prepared from doubly distilled or deionized water. Reagent grade chemicals were used throughout.

NMR Studies. Proton NMR spectra in H_2O were obtained in the CW mode on a Varian HR-220 spectrometer at the ambient probe temperature. The probe temperature was measured using the chemical shifts of a methanol sample. All spectra were time averaged to improve the signal-to-noise ratio. Protein concentrations were ~10% (w/v) in 50 mM phosphate buffer, pH 6.0. In experiments where the metalloenzyme was reconstituted, the correct amount of metal ion to be added was determined from the weight of dry, lyophilized powder of apoprotein. NMR spectra were obtained immediately after reconstitution. Insoluble matter, if any, was removed by Millipore filtration. The reduced enzyme was prepared by addition of small amounts of solid sodium dithionite until the color was bleached. Spectra were scanned in the region from 2.4 to 14.0 ppm downfield from H_2O, which in turn was measured to be 4.89 ppm downfield from TSP subsequently added to the protein solution. Chemical shifts were recorded in ppm from H_2O and converted to the TSP scale using the above correction factor. Values are estimated to be accurate to ±0.1 ppm. Relative intensities were estimated from the weights of traces of the spectral bands.

Chemical Modification Experiments. Diethylpyrocarbonate (DEP, ethoxyformic anhydride) was purchased from Aldrich and assayed using 10 mM imidazole-HCl (pH 6.0) solutions (Dickenson and Dickenson, 1975). The reagent was found to be 82% pure after 7 months of storage at 4 °C.

Protein samples allowed to react with DEP were first diluted to about 2.8 μM (5.6 μM in subunits) with 50 mM sodium acetate buffer (pH 5.9). The Lowry method was employed to check the concentration. The diluted protein solution (3.0 mL) was placed in both the sample and reference quartz cuvettes (1 cm) for study using a Cary 118C recording spectrophotometer. After balancing the absorbance at 242 nm to zero, an appropriate volume of undiluted DEP (5.67 M, corrected for 82% purity) was added to the sample cuvette and mixed well. The increase in absorbance at 242 nm was then monitored until no further change occurred, a process that usually took 2 h because a large excess of DEP was used which must be allowed to hydrolyze before taking a final reading. Under these conditions, the absorbance at 242 nm of DEP added to a cuvette containing only buffer showed an initial increase for 15 min (also observed when DEP is added to buffer in the blank) and then decreased asymptotically to a constant absorbance level reached after 2 h. Because this final absorbance was not zero for higher concentrations of DEP, a blank was run for each concentration of DEP used. For example, 47 mM DEP shows a residual absorbance of 0.009 at 242 nm after 2 h when in the presence of 50 mM acetate buffer (pH 5.9). In a typical experiment the correction for the blank amounted to ~⅓ of the A_{242} reading. By subtracting the value of this blank from the absorbance after 2 h in the reaction with protein, the number of ethoxyformylated histidine residues was calculated using the difference absorption coefficient ϵ_{242} = 3200 M^{-1} cm^{-1} (Ovádi et al., 1967).

A sample of ethoxyformylated native SOD was prepared for 220-MHz NMR studies by adding 0.60 mL of a 1.1 M ethanolic solution of DEP dropwise with stirring to 50 mL of a 33.0 μM solution of native SOD which was buffered at pH 5.7 with sodium acetate. The concentration of DEP was 13 mM in the reaction vessel. An aliquot of this reaction solution was removed and the change in the 242-nm absorbance was monitored as described above. It was found that 1.0 histidine per subunit was modified. The excess reagent, ethanol, and buffer salts were removed by dialysis against doubly distilled water. The protein was then lyophilized. A check of the UV difference spectrum of the redissolved powder showed 1.2 modified histidines.

The titration of apo-SOD with zinc for subsequent reaction with DEP was carried out in the following manner. Apo-SOD, stored in 50 mM acetate buffer (pH 3.7), was dialyzed overnight against this same buffer but with the pH adjusted to 5.9. The concentration of the resulting apo-SOD solution was measured spectrophotometrically and found to be 27.7 μM. This value was checked by the Lowry method which showed the concentration to be 28.0 μM. In a typical experiment, a 10-μL aliquot of 5.7 mM zinc(II) nitrate solution (concentration determined by AA) was then added to 3.56 mL of this apo-SOD solution at room temperature. The final solution contained 0.29 equiv of zinc per subunit of apo-SOD. In similar fashion samples of apo-SOD were prepared that had 0.14, 0.50, 0.58, 0.81, 1.2, 1.7, 6.0, and 116 equiv of Zn^{2+} per subunit. Each of these apo plus Zn^{2+} samples was allowed to react with DEP as described above. Addition of 100 equiv of Zn^{2+} per subunit to native SOD had no effect on the number (i.e., one) of modified histidines. Similarly, the presence of the same concentration of Zn^{2+} in the DEP blank solution did not affect the result.

Results

The proton NMR spectrum of native bovine erythrocyte SOD in the region of the exchangeable proton resonances is

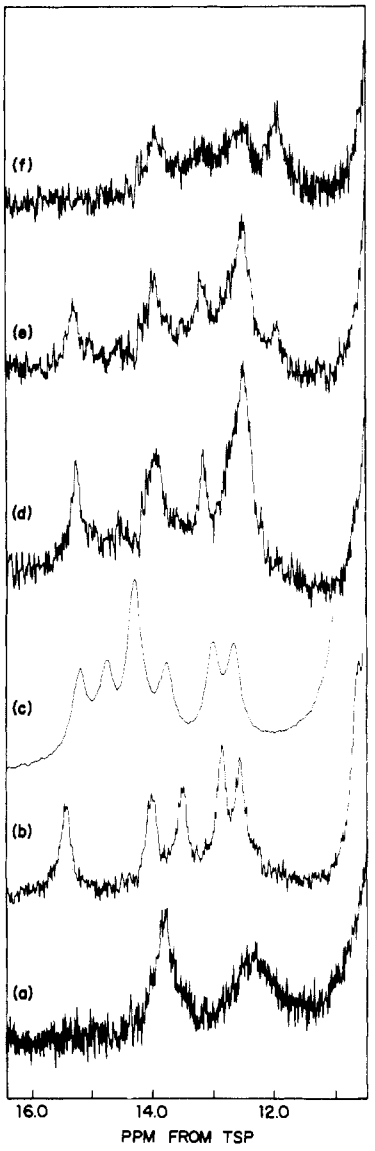


FIGURE 1: The 220-MHz proton NMR spectra of various forms of bovine erythrocyte SOD in H_2O, pH 6 phosphate buffer, 18 °C. Spectra a, b, d, e, and f were obtained under identical conditions (sweep rate 300 Hz/s, 512 scans except for a which was scanned 820 times). Spectrum c was scanned 300 times with a sweep rate of 25 Hz/s. (a) Native oxidized; (b) native reduced; (c) apo + 2.0 equiv of Zn^{2+} per subunit; (d) apo + 1.0 equiv of Zn^{2+}; (e) apo + 0.5 equiv of Zn^{2+}; (f) apoprotein.

shown in Figure 1a. The spectrum consists of a broad band centered at $\delta \sim 12.4$ ppm and a somewhat sharper band at 13.8 ppm. The spectrum of the diamagnetic, reduced enzyme (Figure 1b) reveals at least five readily discernible absorptions. The downfield resonance at 15.4 ppm corresponds to a single proton per subunit. Its absolute intensity was calibrated by comparison with the His-12 N–H resonance at 13 ppm of a pH 4.5 sample of pancreatic ribonuclease of known concentration (Patel et al., 1975). The relative intensity of the sum of the upfield proton resonances at $12.5 < \delta < 14.0$ ppm is five to six times that of the downfield resonance. The relative intensities of these resonances are pH dependent (unpublished results), and all values quoted in this paper are for pH 6.0 solutions. For comparison, the total intensity of tbe broad but detectable resonances in this region for the oxidized protein corresponds to 2.5 protons.

In order to confirm that the observed resonances arise from exchangeable protons, a lyophilized powder of native, oxidized

TABLE I: Summary of Proton NMR Shifts of Bovine SOD in Phosphate Buffer pH 6.0, 18 °C.

Form of SOD	Chemical Shifts (δ, ppm from TSP)					
Native oxidized			13.8			12.4
Native reduced	15.4		14.0	13.45	12.8	12.5
Apo + 1.0 Zn^{2+}	15.2		13.9		13.1	12.5
Apo + 2.0 Zn^{2+}	15.0	14.6	14.1	13.6	12.8	12.5

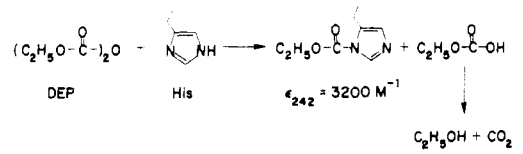


FIGURE 2: The reaction of diethylpyrocarbonate (DEP) with histidine. The adduct has a spectral maximum at 242 nm with an extinction coefficient of 3200 M^{-1} cm^{-1}.

SOD was dissolved in D_2O and reduced with dithionite. The solution pH, uncorrected for the deuterium isotope effect, was 5.7 at 22 °C. After incubation of this sample for 8 h at 47 °C, no resonances were detectable in the region downfield from 11.5 ppm. The protons giving rise to absorbances shown in Figure 1 are all therefore chemically exchangeable with the solvent.

The NMR spectrum of apo-SOD exhibits several broad, poorly resolved features (Figure 1f). This result is attributed to an increased rate of chemical exchange of protons with those of the solvent or to the presence of slow conformational transitions which are removed by metal binding. Addition of up to 1 mol of zinc sulfate per subunit of the apoenzyme sharpens the resonances dramatically, leading to a well-resolved spectrum (Figures 1d and 1e) resembling that of the reduced holoenzyme. Stoichiometric addition of copper(II) acetate to the apo plus 1.0 zinc solution produced a spectrum indistinguishable from that of the native, oxidized SOD.

When more than 1 equiv of zinc was added to the apoenzyme, further NMR spectral changes were observed. The spectrum of apo-SOD in the presence of 2.0 equiv of zinc per subunit is shown in Figure 1c. Addition of cupric ion to this sample had no effect on the NMR spectrum. This result suggests that the second equivalent of zinc prohibits copper from binding in its native site. Addition of zinc beyond 2 equiv per subunit produced no further NMR spectral changes. Table I summarizes the chemical shifts of all the NMR spectral bands.

The ethoxyformylation of histidine residues (Figure 2) in native SOD is shown as a function of initial DEP concentration in Figure 3a. Only one histidine per subunit of native SOD is modified, as reported previously (Stokes et al., 1974). Figure 3e reveals that all eight histidyl residues of apo-SOD may be modified if enough DEP is added initially. The addition of 1 or more equiv of zinc to apo-SOD inhibits seven of the histidines from reacting with DEP (Figure 3b), a result identical with that obtained for the native enzyme. Intermediate results are found for Zn/SOD ratios from 0 to 1, as apparent from Figures 3c and 3d. Assuming no cooperative effects, the following equation may be used to calculate the average number ($\bar{n}_{mod}$) of modified histidines per subunit as a function of the number of equivalents (e) of added zinc per subunit

$$\bar{n}_{mod} = e + 8(1 - e) = 8 - 7e \qquad (1)$$

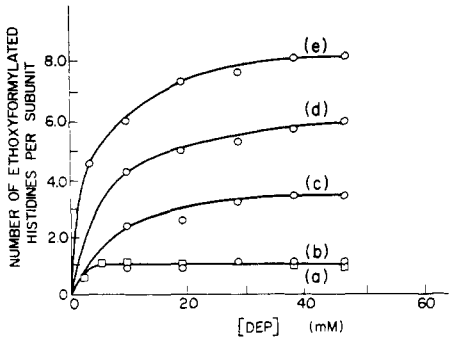


FIGURE 3: Ethoxyformylation of histidine residues in various forms of bovine SOD as a function of initial DEP concentration. (a) Native SOD (□); (b) apo-SOD + 1.30 equiv of Zn^{2+} (○); (c) apo-SOD + 0.65 equiv Zn^{2+}; (d) Apo-SOD + 0.36 equiv of Zn^{2+}; (e) Apo-SOD.

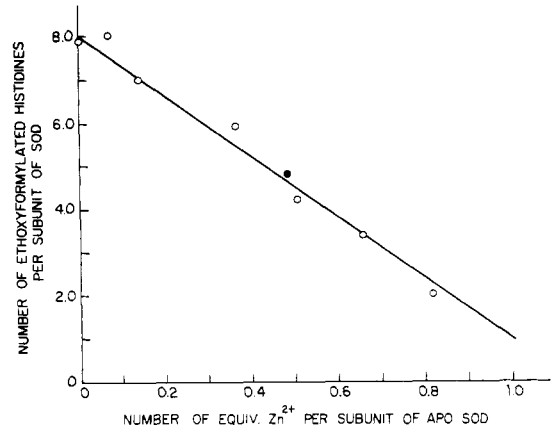


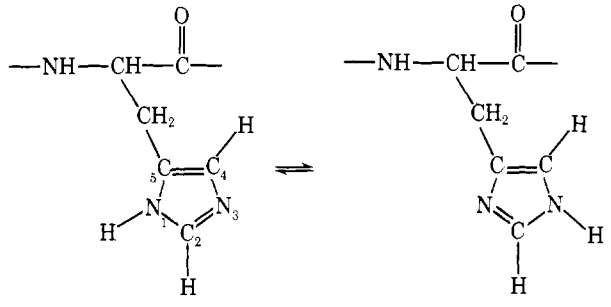
FIGURE 4: Plot of number of modified histidines per subunit of bovine SOD ($\bar{n}_{mod}$) vs. the number of equivalents (e) of added zinc. The line drawn is eq 1. The filled in point was determined in 50 mM phosphate buffer (pH 5.9).

where $0 \leq e \leq 1.0$. As shown in Figure 4, the observed number of modified histidines agrees reasonably well with the values calculated using eq 1. In graphing the figure, correction was made for the 0.07 equiv per subunit of zinc ion left in one of the apo preparations.

As a check on the possible denaturing effects of DEP on the native enzyme, proton NMR spectra of the DEP modified enzyme were recorded in the region of the exchangeable resonances. The spectrum of the ethoxyformylated, reduced enzyme was found to be identical with that depicted in Figure 1b for the unmodified, reduced enzyme. Similar results were obtained for the oxidized enzyme.

Discussion

Nuclear magnetic resonance spectroscopy has been used extensively to study histidine residues in proteins (Markley, 1975). In most of these studies, pulsed Fourier transform methods were employed to investigate the resonances of the C-2 and C-4 imidazole ring protons of the protein dissolved in



D_2O. NMR spectral studies of these ring protons in bovine SOD have revealed that (i) the histidine residues are near the active site (Stokes et al., 1973), a result that came prior to the x-ray work, (ii) metals influence the three-dimensional structure of the protein, and (iii) that at $p^2H \geq 5$, the apoprotein has a well-ordered structure (Fee and Phillips, 1975). The imidazole N-H protons can also be observed in proteins, provided that they are exchanging slowly enough with water. These resonances have been reported between 11.3 and 14.8 ppm for neutral histidine and between 13.1 and 18.0 ppm for protonated histidine (Patel et al., 1975; Robillard and Shulman, 1974a,b). The spectra must be run in H_2O since exchange with deuterated solvent will usually be fast enough, although slow on the NMR time scale, to prohibit the observation of the N-H proton resonances. Histidine residues must either be hydrogen bonded or sufficiently protected by the protein structure from rapid exchange with H_2O in order for their N-H protons to be observed.

The NMR spectrum of reduced bovine erythrocyte SOD exhibits several resonances downfield of 12.5 ppm (Figure 1, Table I) in the region expected for the exchangeable N-H protons of histidine. These resonances are drastically affected when the protein is in its native, oxidized state. The broadening or disappearance of the low-field resonances in oxidized SOD is attributed to paramagnetic relaxation by the cupric ion. The possibility that some of the peaks are shifted upfield of 11.5 ppm into the region of numerous overlapping resonances or are exchanging rapidly with the solvent to produce undetectably broad signals in the spectrum of oxidized SOD is not considered likely. In order to produce the required shifts ($\geq$1 ppm) or increases in exchange rate ($\Delta\nu_{1/2} \sim 40$ Hz to ≥ 150 Hz), a large change in the local conformation and/or hydrogen bonding of the residues exhibiting the proton resonances would have to accompany oxidation of copper(I) to copper(II). Since, as discussed below, the presence of only zinc and no copper at all yields a spectrum similar to that of the reduced protein, any conformational changes that occur upon oxidation of cuprous SOD are unlikely to produce the large changes observed in the NMR spectrum.

Paramagnetic broadening of the proton resonances will arise from scalar and dipole-dipole interactions for the ligated histidines and from dipole-dipole interactions alone for amino acids not bonded to the copper(II) ion. A calculation[2] assuming a correlation time of 10^{-8} s for the dipole-dipole interaction shows that nuclei farther than 11 Å away from the paramagnetic copper center should remain detectable in our spectra. In the absence of knowledge of the side-chain distances from the copper atom, the published α-carbon coordinates (Richardson et al., 1975b) were used to approximate those residues likely to exhibit paramagnetic broadening effects. These include seven histidine (the six copper and zinc ligands and His-41), two lysines (Lys-120 and Lys-134), three asparagines (Asn-63, Asn-84, and Asn-137), and Arg-141. Although we

[2] $\Delta\nu_{1/2} = (\hbar^2/20\pi)\gamma_H^2\gamma_e^2(1/r^6)[4\tau_c + (3\tau_c/(1+\omega_H^2\tau_c^2))]$; $(\omega_{Cu}^2\tau_c^2 \gg 1)$, where ω_H and ω_{Cu} are the ^1H and Cu(II) electron Larmor frequencies, respectively, γ_H and γ_e are the gyromagnetic ratios of the proton nucleus and copper electron, respectively, r is the distance between these magnetic centers, and τ_c, the correlation time, is given by $1/\tau_c = (1/\tau_R) + (1/\tau_e)$, where τ_R is the rotational correlation time and τ_e the electron spin relaxation time (Dwek, 1973). The value for τ_R is expected to be $\sim 10^{-8}$ s, and τ_e lies between 10^{-8} and 10^{-10} s for cupric complexes (Poupko and Luz, 1972). τ_c was therefore conservatively taken to be 10^{-8} s, corresponding to maximum paramagnetic line broadening.

cannot definitely rule out the possibility that the exchangeable proton resonances displayed in Figure 1 arise from Arg, Lys, or Asn, it should be noted that the chemical shifts of these residues usually appear at δ 7 ppm. Unusually strong hydrogen bonding would be required to shift them downfield of 12.5 ppm. We therefore favor assignment of these resonances to the N-H protons of coordinated histidine residues and to His-41, the latter being buried in the β-barrel structure of the protein. The remaining histidine, His-19, is known from the x-ray structure to be exposed to solvent and its N-H resonance is not expected to be visible. The fact that the NMR spectrum of reduced holoenzyme is unchanged by reaction with DEP demonstrates that His-19 is not one of the six or seven absorptions that appear. Further NMR work should permit assignment of these bands to specific amino acids. It should, however, be emphasized that conclusions reached in this paper are independent of such assignments. The fact that the proton chemical shifts and/or exchange rates are sensitive to structural perturbations is sufficient for their use as reporters of structural changes in the protein.

The fact that ethoxyformylation of native SOD results in the chemical modification of only one histidine per subunit is nicely consistent with the x-ray structural results. The reactivity of metal complexed histidines toward DEP is expected to be greatly diminished compared with free histidines because the lone pair of electrons on the nucleophilic pyridine nitrogen atom of the imidazole group is coordinated. Indeed, this point has been demonstrated in work with model compounds (Ludewig et al., 1975). Since His-41 is buried, the one that is modified by DEP is most likely His-19.

The utility of DEP as a protein modification reagent depends upon a number of factors. It is only slightly soluble in water and a 30 mM solution is considered saturated. Its stability is a function of pH and buffer. A half-life of 24 min was previously found in 60 mM potassium phosphate buffer pH 6.0 at 25 °C, while the half-life of DEP in 0.1 M Tris-Cl buffer pH 7.5 is 1.3 min (Berger, 1975). We measure the half-life in 50 mM sodium acetate buffer pH 5.9 to be 19 min. The extent of modification of a protein is therefore dependent on the total amount of reagent added and on the ratio of the rate constants for the competing hydrolysis of DEP and the ethoxyformylation of protein functional groups. It is therefore often necessary to use larger concentrations of DEP than are normally employed in chemical modification studies using more stable reagents.

It should be noted that DEP is not specific for histidine. It has been shown to react with several amino acid functional groups in proteins. These include phenolate, carboxylate, sulfhydryl, α- and ϵ-amino and guanidino groups (Burstein et al., 1974). Only histidine, however, reacts with DEP to give a product with a characteristic absorbance in the region near 242 nm. Ethoxyformyltyrosine gives a negative absorbance at 278 nm (ϵ_{278} = 1310 M^{-1} cm^{-1}) in difference spectra of modified proteins. It has been reported that this absorbance interferes with the quantification of ethoxyformylated histidines (Burstein et al., 1974). No band at 278 nm was found in any of the difference spectra for the various enzyme derivatives during this investigation.

There is also evidence to show that DEP reacts with sulfhydryl groups in the presence of carboxylate buffers to form a product which absorbs at 242 nm (Garrison and Himes, 1975). This reaction therefore may interfere with the use of DEP to quantify histidine residues when the reaction is performed in carboxylate buffers. This interference probably does not occur for SOD because a control experiment run in 50 mM phosphate buffer (pH 5.9) gave the same results as in acetate buffer. Moreover, there is only one cysteine residue present per subunit and it is known to be buried from x-ray diffraction studies (Richardson et al., 1975a). Nonetheless, if it were to react in the manner suggested by Garrison and Himes (1975), it would introduce an error of only +0.3 ethoxyformylated histidines per subunit.

As seen from Figure 3e, all eight histidines per subunit of apo-SOD are modified by DEP. It was previously reported (Stokes et al., 1974) that four histidines of apo-SOD became ethoxyformylated under the conditions where the DEP and apo-SOD concentrations were 3.5 mM and 130 μM, respectively. It appears likely from our results that insufficient amounts of DEP were added to complete the reaction. On the other hand, it is possible that the 47 mM concentration of DEP used in the present work constitutes denaturing conditions for apo-SOD, thereby increasing the reactivity of normally buried histidines. Although we believe it to be unlikely this possibility can not be ruled out with the data collected here.

The NMR spectrum of apo-SOD consists of several broad, weak resonances. Addition of up to 1 equiv of zinc per subunit of apo-SOD dramatically restores the N-H proton NMR spectrum to a pattern closely resembling that of the reduced enzyme. This result suggests that zinc alone is capable of structuring the active site into a conformation resembling that of the native protein. The ethoxyformylation results (Figure 3) reinforce this interpretation. It is assumed, although not known for certain, that zinc occupies its natural site when bound in 1:1 stoichiometry to the apoprotein. In either case, the failure of all but one histidine to react with DEP in the presence of 1 or more equiv of zinc strongly suggests that much of the native structure of the active site of the protein has been restored. The lack of reactivity of histidine residues not bonded to zinc reflects their steric inaccessibility to DEP.

At zinc concentrations between 0 and 1 equiv per subunit, the number of ethoxyformylated histidines can be nicely accounted for statistically (Figure 4). The addition of larger amounts of zinc (100 equiv) did not affect the DEP modification results. The NMR spectrum, on the other hand, exhibits further changes upon addition of up to 2 equiv of zinc per subunit, beyond which the pattern remains constant. The number of clearly discernible resonances in the apo plus 2 Zn^{2+} spectrum is greater than that of the apo plus 1 Zn^{2+} spectrum (Figure 1). One new resonance could arise if zinc occupies both metal sites but His-61 is no longer bridging the two metal ions. The chemical shifts of the N-H resonances in the reduced native and apo plus 2 Zn^{2+} spectra are quite similar (Table I). The additional resonance at 14.6 ppm might be a proton on His-61. The conclusion that the apo plus 2 Zn^{2+} sample does have zinc in both metal sites is supported by the fact that stoichiometric addition of copper did not restore the NMR spectrum of the native enzyme. Addition of copper to the apo plus 1 Zn^{2+} sample did produce the NMR spectrum of the oxidized holoenzyme.

Utilization of the histidine N-H NMR spectra to elucidate the structure of the active site and features of the enzyme mechanism of bovine SOD should be possible once the individual resonances are assigned. The present results have been satisfactorily interpreted in terms of the 3.0 Å resolution x-ray data for crystalline SOD. Further NMR and x-ray work should permit additional structural correlations.

Acknowledgments

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References

Berger, S. J. (1975), *Anal. Biochem. 67,* 428.
Burstein, Y., Walsh, K. A., and Neurath, H. (1974), *Biochemistry 13,* 205.
Chlebowski, J. F., and Coleman, J. E. (1976), in Metal Ions in Biological Systems, Siegel, H., Ed., New York, N.Y., Marcel Dekker, pp 1-140.
Dickenson, C. J., and Dickenson, F. M. (1975), *Eur. J. Biochem. 52,* 595.
Dunn, M. F. (1975), *Struct. Bonding (Berlin) 23,* 61.
Dwek, R. A. (1973), Nuclear Magnetic Resonance in Biochemistry, London, Oxford University Press.
Fee, J. A. (1973), *Biochim. Biophys. Acta 295,* 87.
Fee, J. A., and Phillips, W. D. (1975), *Biochim. Biophys. Acta 412,* 26.
Fee, J. A., and Ward, R. L. (1976), *Biochem. Biophys. Res. Commun. 71,* 427.
Fielden, E. M., Roberts, P. R., Bray, R. C., Lowe, D. J., Mautner, G. N., Rotilio, G., and Calabrese, L. (1974), *Biochem. J. 139,* 49.
Forman, H. J., and Fridovich, I. (1973), *J. Biol. Chem. 248,* 2645.
Fridovich, I. (1973), *Biochemistry 12,* 823.
Fridovich, I. (1975), *Annu. Rev. Biochem. 44,* 147.
Garrison, C. K., and Himes, R. H. (1975), *Biochem. Biophys. Res. Commun. 67,* 1251.
Hodgson, E. K., and Fridovich, I. (1975), *Biochemistry 14,* 5294.
Keele, B. B., McCord, J. M., and Fridovich, I. (1971), *J. Biol. Chem. 246,* 2875.
Klug-Roth, D., Fridovich, I., and Rabani, J. (1973), *J. Am. Chem. Soc. 95,* 2786.
Lippard, S. J., Burger, A. R., Ugurbil, K., Valentine, J. S., and Pantoliano, M. W. (1977), *Adv. Chem. Ser.* (in press).
Ludewig, M., Frohne, M., Marquardt, I., and Hanson, H. (1975), *Eur. J. Biochem. 54,* 155.
Markley, J. L. (1975), *Acc. Chem. Res. 8,* 70.
McCord, J. M., and Fridovich, I. (1969), *J. Biol. Chem. 244,* 6049.
Misra, H. B., and Fridovich, I. (1972), *J. Biol. Chem. 247,* 3170.
Ornstein, L. (1964), *Ann. N.Y. Acad. Sci. 121,* 321.
Ovádi, J., Libor, S., and Elödi, P. (1967), *Acta Biochim. Biophys. Acad. Sci. Hung. 2,* 455.
Patel, D. J., Canuel, L. L., Woodward, C. W., and Bovey, F. A. (1975), *Biopolymers 14,* 959.
Pouko, R., and Luz, Z. (1972), *J. Chem. Phys. 57,* 3311.
Richardson, J. S., Thomas, K. A., and Richardson, D. C. (1975b), *Biochem. Biophys. Res. Commun. 63,* 986.
Richardson, J. S., Thomas, K. A., Rubin, B. H., and Richardson, D. C. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1349.
Robillard, G., and Shulman, R. G. (1974a), *J. Mol. Biol. 86,* 519.
Robillard, G., and Shulman, R. G. (1974b), *J. Mol. Biol. 86,* 541.
Rotilio, G., Calabrese, L., Bossa, F., Barra, D., Agró, A. F., and Mondovi, B. (1972), *Biochemistry 11,* 2182.
Steinman, H. M., Naik, V. R., Abernathy, J. L., and Hill, R. L. (1974), *J. Biol. Chem. 249,* 7326.
Stokes, A. M., Hill, H. A. O., Bannister, W. H., and Bannister, J. V. (1973), *FEBS Lett. 32,* 119.
Stokes, A. M., Hill, H. A. O., Bannister, W. H., and Bannister, J. V. (1974), *Biochem. Soc. Trans. 2,* 489.