BBA 37193

THE BEHAVIOR OF HOLO- AND APO-FORMS OF BOVINE SUPEROXIDE DISMUTASE AT LOW pH

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SUMMARY

1. Holo-superoxide dismutase from bovine erythrocytes has been shown to undergo a reversible structural modification in the pH 3–5 range.
2. The spectral alterations observed on changing from neutrality to pH 3 were: a slight attenuation of the 680 nm absorbance; the loss of the 450 nm shoulder, apparent in the optical spectrum of the native protein; and a new band appeared at 330 nm. The circular dichroism at 600 nm was essentially lost while a weak negative band appeared at approx. 380 nm and a positive band at 310 nm.
3. The EPR spectrum was also modified on changing from the native to the low pH form: $A_\parallel$ increased from ≈ 130 to ≈ 150 G, $g_\parallel$ remained unchanged at ≈ 2.27, and g_m decreased from ≈ 2.09 to ≈ 2.08. The apparent linewidth remained essentially constant.
4. High resolution (220 MHz) PMR spectra of holo- and apoproteins revealed that the metals influence the three-dimensional structure of the protein.
5. PMR studies indicated that at pH 3 the apoprotein existed almost entirely in a random coil form and that it assumed a compact well-ordered structure on returning to neutral pH. The holoprotein maintained a compact, apparently dimeric, structure even at pH 3.

INTRODUCTION

Superoxide dismutase has a molecular weight of 31 000 and consists of two chemically identical subunits [1, 2]. Each subunit binds a Zn^{2+}-Cu^{2+} pair, the two metal ions are in very close proximity [3–5], and they form the active centers of the enzyme. The oxidation-reduction potential of the Cu^{2+} has been determined in the pH range 4–8.7 [6]. Above pH 5.5 the redox potential decreases 0.059 V per pH unit,
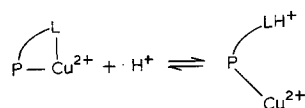
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while below pH 5.5 there is very little dependence of E_m on pH. The half reaction above pH 5.5 can be described by:

$$P-\overset{\overset{L}{|}}{Cu}{}^{2+} + e^- + H^+ \rightleftharpoons P-\overset{\overset{LH^+}{}}{Cu}{}^+$$
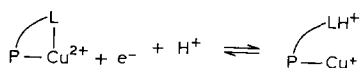
while below pH 5.5 the ligand L may be protonated in the absence of reduction:

$$P-\overset{\overset{L}{|}}{Cu}{}^{2+} + \cdot H^+ \rightleftharpoons P\overset{LH^+}{\underset{Cu^{2+}}{\diagup\diagdown}}$$

Our search for this equilibrium has led us to discover that both holo- and apoenzymes undergo conformational changes in the pH 3–5 range. The spectral and sedimentation changes accompanying these alterations of the protein's structure are described.

EXPERIMENTAL SECTION

Materials. Superoxide dismutase was prepared by the method of McCord and Fridovich [7] and apoprotein was prepared as previously described [4, 7]. ^2H_2O 99.9% was obtained from BioRad Laboratories. All other chemicals were of highest commercial quality and where appropriate double distilled water was used.

Instrumentation. Routine pH measurements were made using a Heath Model EU-302A pH meter and a Fisher combined glass/Ag/AgCl electrode. The pH of PMR samples were measured with a Beckman pH meter and a specialized combination glass electrode. For solutions containing primarily ^2H_2O the activity of the $^2H^+$ was obtained from p^{2}H = pH meter reading + 0.4 [8].

EPR spectra were obtained with a Varian E-9 EPR spectrometer operating at X-band with 100 kHz field modulation. Spectra were recorded at temperatures near 100 °K employing a device similar to that described by Hansen et al. [9] for temperature control.

PMR spectra were acquired with a Varian FFT 220 MHz high resolution spectrometer operating in the continuous wave or in the Fourier transform mode at 220 MHz. Sample temperature was maintained at 37 ± 1 °C in the described experiments. The signal to noise characteristic in the aromatic region of the spectra was enhanced by repeated collection of free induction decay patterns. All data manipulation was carried out with the Varian 620I computer. The general procedures have been described elsewhere [10].

Optical spectra were recorded with a Cary Model 14 dual beam spectrophotometer, and circular dichroism spectra were acquired with a Cary 60A recording spectropolarimeter.

Sedimentation coefficients were measured using a Beckman Model E analytical ultracentrifuge operating at 60 000 rev./min, and cells producing a synthetic boundary were used.

Preparation of samples for PMR measurements. Samples of native or apoprotein were exchanged into ^2H_2O by one of two methods: (a) Dilution with ^2H_2O and slow concentration under a stream of dry N_2, or (b) freeze-drying followed by

dissolution in ^2H_2O. No differences were observed in samples prepared by either procedure. Further, it should be emphasized that in properly treated samples of holoprotein, exchange into ^2H_2O by either of the described techniques effects absolutely no change in the observed EPR spectrum (cf. ref. 11).

RESULTS

When the pH of an approx. 1 mM solution of superoxide dismutase is lowered from neutrality to approx. 3, the blue-green color of the solution changes to one of clear cerulean blue. This is a reversible transformation as returning the pH to neutrality will restore the blue-green color. The changes in the visible optical spectrum are shown in Fig. 1 which describes some results of the titration from low to high pH
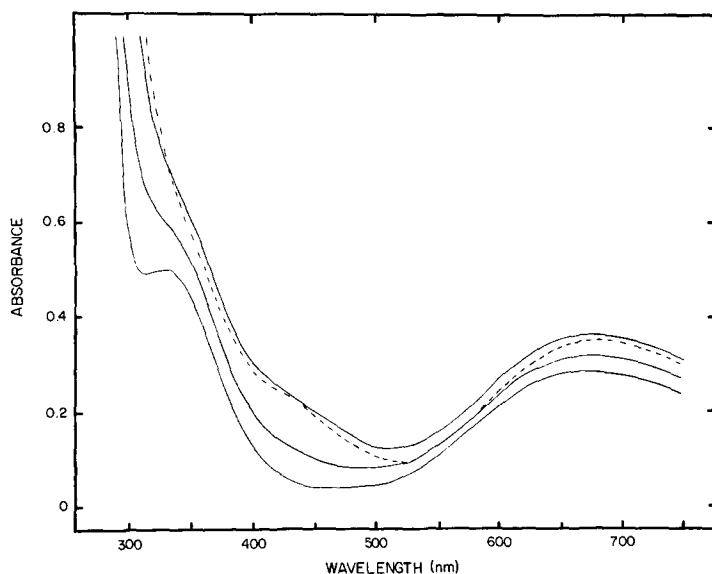

Fig. 1. Effect of pH on the visible optical spectrum of superoxide dismutase. Solid lines (————): Lower, holoenzyme adjusted from neutrality to a stable pH of 3.2; Middle, pH adjusted from 3.2 to 3.65 (stable); Upper, pH adjusted from 3.65 to 3.80. Dashed line (— — —): After the last pH adjustment the sample was allowed to stand overnight, and it was then centrifuged to remove a small amount of insoluble denatured protein before the spectrum was recorded. Final pH 4.2. This spectrum corresponds to that of native protein.

and serves to demonstrate the reversibility. The spectral changes which occur on going to low pH are: approximately a 15% attentuation of absorbance in the region of 680 nm, a complete loss of the strong shoulder at 450 nm, the loss of strong absorbance below 350 nm, and the appearance of a new but weaker absorption band at approx. 330 nm. The change in color from blue-green to blue appears due to the loss of the band near 450 nm.

Fig. 2 demonstrates the changes which occur in the visible circular dichroism spectrum on lowering the pH. The strong positive band at 600 nm and presumably
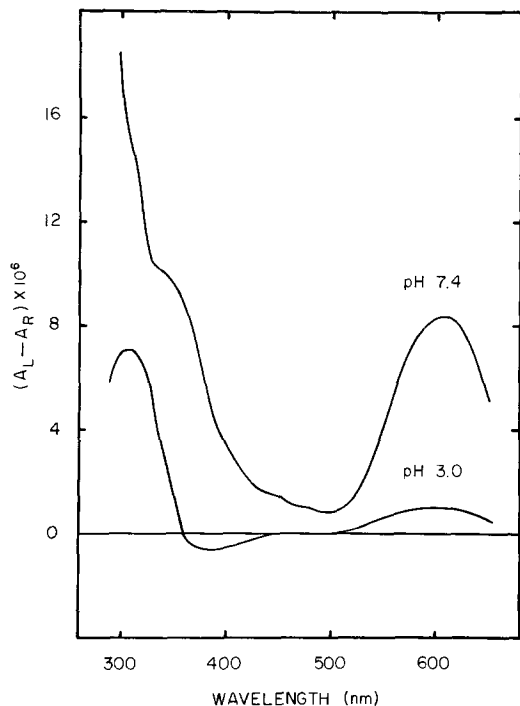
Fig. 2. Circular dichroism spectra of superoxide dismutase at pH 7.4 and 3.0.

the negative 750 band[*] are both nearly lost. Indeed, the apparent dichroism in this region may only be due to some remaining high pH form of the molecule. Further, the strong positive shoulder at 350 nm is replaced by a weaker band near 310 nm. The apparent but weak negative dichroism near 380 nm would be obscured if all enzyme was not converted to the low pH form. Thus, the 330 nm band in the optical spectrum may consist of one positively dichroic band below 330 nm and a negative band above 330 nm.

The changes which occur in the low temperature EPR spectrum are shown in Fig. 3. On lowering the pH, as indicated, the negative spectrum, having the parameters $A_{\parallel} \simeq 130$ G, $g_{\parallel} \simeq 2.27$, and $g_m \simeq 2.09$, was modified to one having the parameters, $A_{\parallel} \simeq 150$ G, $g_{\parallel} \simeq 2.27$ and $g_m \simeq 2.08$. The low pH spectrum also appears to be less rhombic [12, 13] than the neutral pH spectrum. At the lowest pH attempted with native protein a small amount of denaturation appeared to occur as evidenced by the amplified portion of the spectrum recorded at pH 2.9. Again, the EPR spectrum can be almost entirely returned to that characteristic of the native form by adjusting the pH toward neutrality. The lack of complete reversibility from pH 2.9 is evident in Fig. 3 by the slightly lower resolution of the superhyperfine structure just above 3200 G, but this represents the modification of only a minor fraction of the total protein[**].

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[*] The circular dichroism spectrum above 700 nm has been described in the advertising literature of Varian Associates: Circular 1A-621 (1972). The spectrum exhibits a negative band centered at approx. 750 nm.

[**] The above results may be compared with those reported earlier by other authors [34].
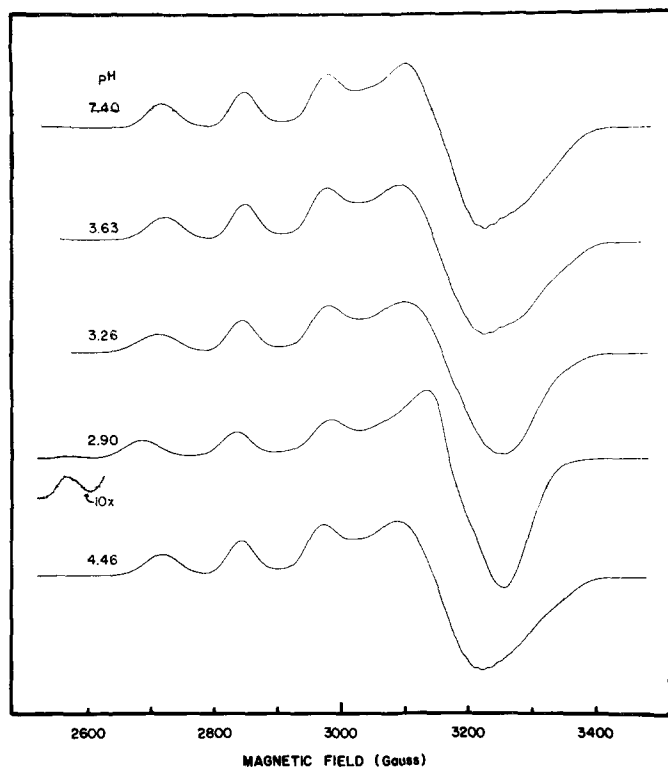
Fig. 3. Effect of pH on the EPR spectrum of superoxide dismutase. The experiment was carried out as follows: 1.6 ml, 1.24 mM dismutase in 10^{-4} M potassium phosphate buffer, pH 7.4, was titrated with 1 M H_3PO_4. During titration the sample was stirred. 84 μl of acid was required to lower the pH to 2.90. When the pH had stabilized at a desired value both optical and EPR spectra were recorded using the same sample. This required about 10 min after which the pH had generally risen less than 0.1 unit. After titration to pH 2.90 the pH was returned to 4.46 by addition of NaOH. EPR spectra were recorded at 100 °K with the following instrumental settings: Modulation amplitude, 10 G; time constant, 0.1 s; field sweep, 500 G/min.

In spite of its essential reversibility the low pH transition of native superoxide dismutase is accompanied by considerable hysteresis, as demonstrated by Fig. 4 in which relative optical and EPR changes in the pH region of the transition are presented. The underlying basis of the hysteresis is probably related to the slowness of the pH-dependent structural transition on going from high to low pH. Thus, after addition of acid at room temperature several minutes are required to achieve a nominally stable pH (up to 30 min), but the time course of the pH change, particularly over longer periods, has not been studied. In the several experiments which were carried out, a comparable time dependence was not observed on titration from low to high pH.

The question of whether the native dimeric molecule became dissociated at low pH values was studied by measuring the effective sedimentation coefficient of holoenzyme in 0.1 M NaCl at pH 4, 3.5 and 3; S_{eff} decreased only from 2.8 S at pH 4 to 2.4 S at pH 3 (Fig. 5). This strongly suggests that significant dissociation into monomers did not occur.
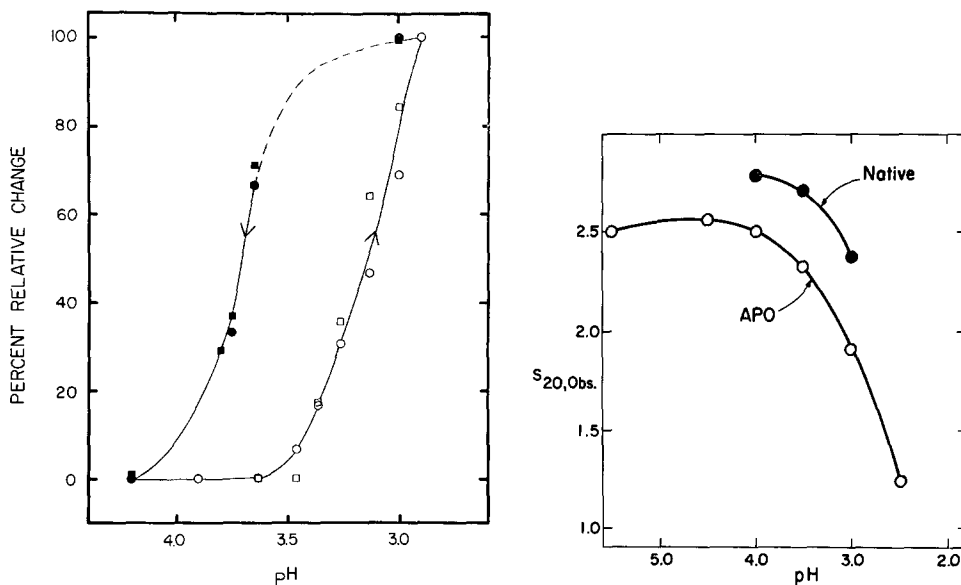
Fig. 4. Demonstration of the hysteresis effect in the low pH titration of superoxide dismutase. The experiment described in the legend of Fig. 3. Changes in absorbance at 450 nm in the down (○) and (●) up titration. Increase in EPR signal intensity at 3250 G in the down (□) and up (■) titration. Corrections for volume changes were made. The lines are not derived from any theory.

Fig. 5. The effect of pH on the sedimentation coefficients of holo- and apo-superoxide dismutases. The concentration of protein was 8 mg/ml in 0.1 M NaCl, the temperature was 20 °C, and the pH of each sample was measured after standing overnight at room temperature. Plots of ln $r(t)/r(t_0)$ vs t were perfectly linear in all cases. The ratio $r(t)/r(t_0)$ was obtained using a comparator. No corrections to the observed sedimentation coefficients were made.

High resolution PMR spectra (Fig. 6) of holoprotein taken in the range p^2H 6.9–3.4 demonstrate that the three-dimensional structure of the polypeptide chains is substantially preserved even at p^2H 3.4. There are, however, changes in the spectrum which suggest conformational alterations on going to low pH; for examples, the sharpening of the resonance envelopes near 1, 7.5, and 8.5 ppm which probably reflect overall changes in protein structure as well as the specific changes occurring near the metal binding site.

The behavior of the apoprotein has also been studied by PMR spectroscopy (Fig. 7). Above $p^2H \approx 5$ the apoprotein possesses a compact conformation evidenced by the broad resonance envelopes in both aromatic and aliphatic regions. Its overall conformation, however, appears to differ somewhat from that of the holoprotein. This is best illustrated by comparing the spectra of holo- and apoproteins in the 6.5–7.5 ppm region. Here the spectrum of apoprotein has a distinct doublet structure while only a single broad line can be seen in the spectrum of holoprotein. The generally higher resolution observed in the apospectrum compared to the holo spectrum may arise from two sources: (a) broadening of resonances by the paramagnetic Cu^{2+} [14], and (b) a generally "looser" or a conformationally more dynamic structure resulting in a greater averaging of anisotropic magnetic interactions [15]. Previous
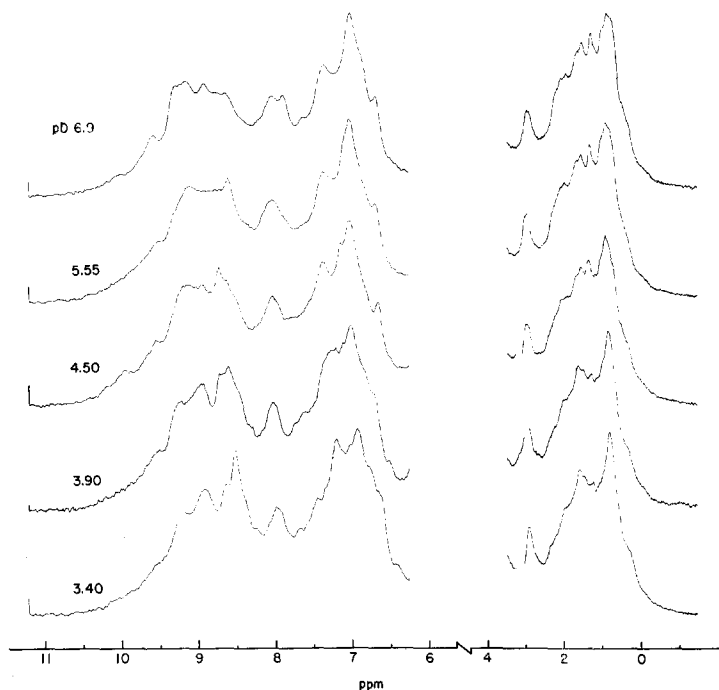
Fig. 6. The effect of pH on the 220 MHz PMR spectrum of superoxide dismutase. The concentration of the samples used was approx. 60 mg/ml. pH adjustments were made by adding small amount of NaO^2H or ^2HCl with rapid stirring. The frequency scale based on the HO^2H line which was determined to be 1020 Hz downfield from 2,2-dimethyl-2-silapentane-5-sulforate at 37 °C. The aliphatic region (1–4 ppm) was recorded in the continuous wave mode. The aromatic region (6–11 ppm) was recorded in the FT mode and recorder output is normalized to the highest peak in this spectral region. The large HO^2H signal and spinning side bands have been deleted.

results on the reconstitution of holoprotein from apoprotein and metal ions have been interpreted in terms of a structural difference between the two forms [3].

When the pH of a solution of apoprotein is lowered from p^2H 5.7 to 4.0 the PMR spectrum changes dramatically (Fig. 7) from that characteristic of a compact-folded structure to one characteristic of the random coil structure [16, 17]. Measurement of the sedimentation coefficient of apoprotein over the pH range 5.5–2.5 revealed a decrease of S_{eff} from 2.56 S at pH 4.5 to 1.29 S at pH 2.5 (Fig. 5). Clearly such a large change in S_{eff} is consistent with the occurrence of monomerization, and the PMR spectrum suggests dissociation of the subunits into randomly coiled peptide chains.

It is noteworthy that readily detectable changes do not occur in the optical spectrum above 250 nm or in the circular dichroism spectrum above 200 nm. We have thus used PMR to examine the reversibility of the low pH transition of apo-protein. When base is added to a concentrated (60 mg/ml) solution of apo-superoxide dismutase so as to adjust the pH from say 3 to 5, there is an immediate and apparently irreversible precipitation of the protein from solution. If, however, base is added so as to effect only very small increases in pH and this is done slowly then no precipitation
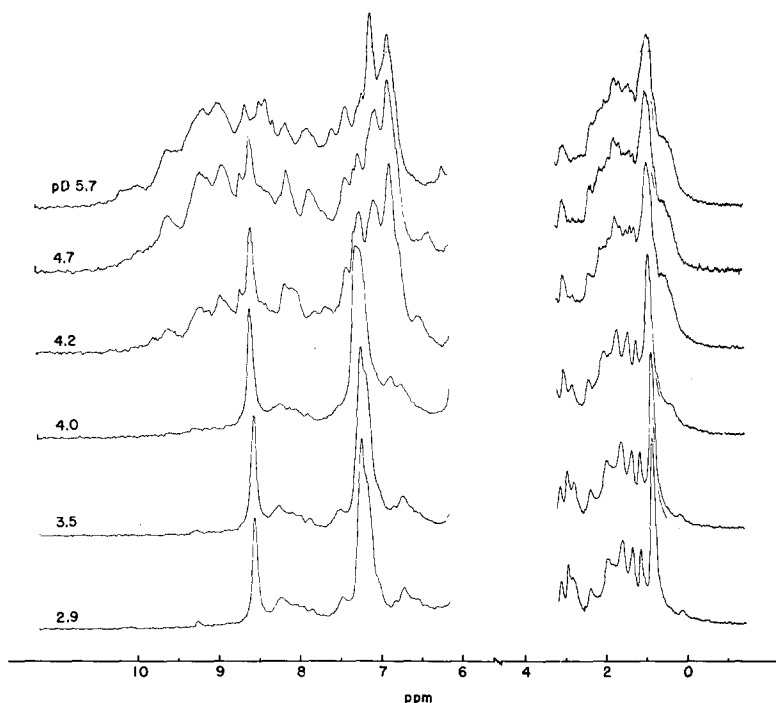
Fig. 7. Effect of lowering p^2H from 5.7 to 2.9 on the 220 MHz PMR of apo-superoxide dismutase. Conditions and procedures similar to those described in legend to Fig. 6.

occurs and the transition seems to be substantially reversed; the results of a PMR experiment are shown in Fig. 8. The reversibility can be seen most readily by comparing the p^2H 5.7 spectrum with the p^2H 5.9 spectrum in Fig. 8. Note that in the field range 7.5–1.0 ppm these two spectra are substantially the same. Below 7.5 ppm the apparent differences between these two spectra are believed to be due to a greater portion of N-H exchange to N-^2H in the p^2H 5.7 spectrum [18]. This deduction is justified by comparing the p^2H 5.7 spectrum from Fig. 7 with the p^2H 5.7 spectrum in Fig. 8. The latter was obtained from a dilute sample of apoprotein which had been brought to pH 3 and returned to neutral pH in water prior to being concentrated and exchanged into ^2H_2O and is thus directly comparable to the p^2H 5.7 spectrum shown in Fig. 7. Clearly the low pH transition is reversible if precautions are taken to avoid precipitation either by increasing the pH very slowly or by using a dilute solution.

To further test the supposition that the individual polypeptide chains assume the properties of random coils at low pH we have computed a "random coil" spectrum, utilizing published line positions and widths [17] in conjunction with the amino acid composition [1, 2]. Somewhat arbitrarily the resonance position of the protonated His C-4 proton was taken as 7.30 ppm, just below the published value of 7.26 ppm for the aromatic protons of phenylalanine. Bradbury and Scheraga [19] have reported that the C-4 proton of protonated histidine was observed near 7.3–7.4 ppm, and McDonald and Phillips [20] in discussing the His C-4 resonances of ferrocytochrome c indicate considerable latitude in the position of this proton. In general, the correspondence between the calculated and observed spectra were good. The peak positions
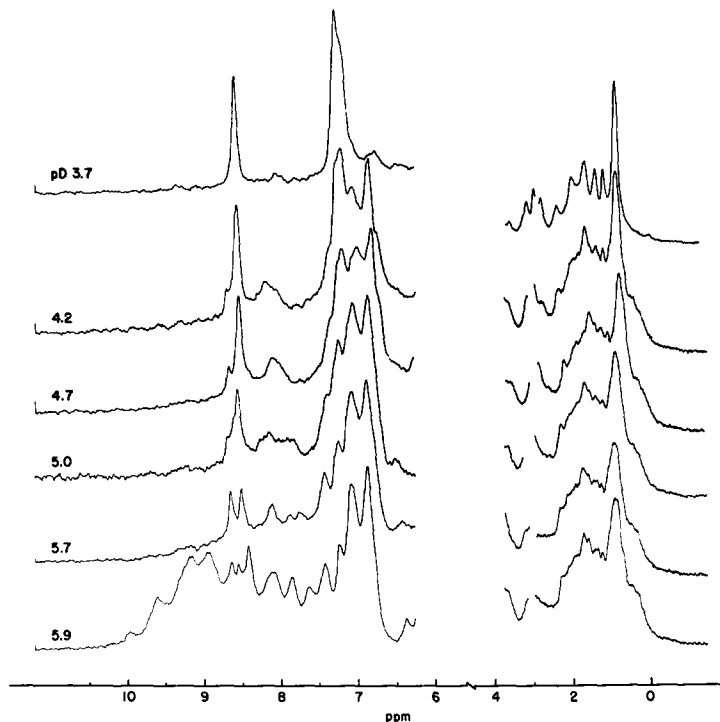
Fig. 8. Effect of raising p²H from 3.7 to 5.7 on the 220 MHz PMR spectrum of apo-superoxide dis-mutase. Conditions and procedures as in Fig. 6. The lower spectrum was obtained from a sample which had been adjusted to pH 3 in water followed by readjustment to pH 6 and exchanged into ²H₂O by the freeze-dry dissolution technique.

and relative intensities of the two spectra are compared in Table I. It can be seen that the resonance positions are only slightly different and that the relative intensities are comparable, particularly in the aromatic region. The small differences in resonance positions may reflect the fact that the parameters used were for neutral p²H at elevated temperatures [17]. The small differences in relative intensities suggest that a complete randomization of the polypeptide chain had not occurred.

DISCUSSION

Holoprotein

The above results demonstrate clearly that holo-superoxide dismutase under-goes a structural change in the immediate vicinity of the Cu^{2+} on lowering the pH from neutrality to approx. 3, and this results in modifications of the optical, circular dichroism, and EPR spectra. A possible interpretation of the low pH behavior can be made in terms of the recently determined structure of superoxide dismutase.

The Richardsons' group has demonstrated by X-ray crystallographic measure-ments that an imidizolium anion serves as a bridging ligand between Zn^{2+} and Cu^{2+} [21]. The constant for the ionization of neutral imidazole to its anion is approx. 14 [22] while the analogous ionization constant for the imidazole ring of dihistidyl-Cu^{2+}
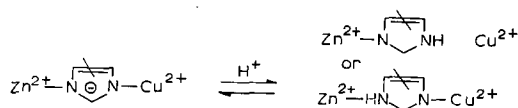
TABLE I

COMPARISON OF OBSERVED AND CALCULATED[*] PMR SPECTRA OF SUPEROXIDE DISMUTASE AT LOW pH

[*] Parameters used were: Residue, intensity, resonance (ppm), full line width at half height (Hz); Leu-CH$_3$, 40, 0.886, 15; Ile-CH$_3$, 30, 0.832, 20; Val-CH$_3$, 35.2, 0.932, 17; Thr-CH$_3$, 18.7, 1.23, 16; Ala-CH$_3$, 16.7, 1.41, 18; Lys-δCH$_2$ + βCH$_2$, 13.3, 1.68, 30; Met-CH$_3$, 30, 2.06, 10; Tyr-ortho H, 11.8, 6.82, 17; Tyr-meta H, 11.8, 7.09, 17; Phe (aromatic), 16.7, 7.26, 30; His C-4, 10, 7.30, 10; His C-2, 10, 8.56, 10. Except for His C-4 al′ parameters taken from ref. 17. A Lorentzian line shape was assumed.

Observed		Calculated		Assignment	Number of contributing residues per subunit [1]
Line position	Relative intensity	Line position	Relative intensity		
Aliphatic region					
0.77	100	0.92	100	CH$_3$- of Leu, Ile, Val	8, 9, 15
1.09	40	1.23	35	Thr-CH$_3$	12
1.32	41	1.41	24	Ala-CH$_3$	9
1.57	44.7	1.68	19.4	Lys-δ, β CH$_2$	10
1.94	34.8	2.06	11.4	Met-CH$_3$	1
Aromatic region					
6.75	20	6.94	10.4	Tyr (ortho)	1
7.05(ws)		7.10		Tyr (meta)	1
7.15(s)	81	7.27		Phe (aromatic)	4
7.22	100	7.30	100	His C-4	8
8.55	58	8.56	61	His C-2	8

has been measured to be approx. 11.7 [23, 24]. The latter pK_a is sufficiently high to account for the linear decrease of the midpoint potential, E_m, of the Cu^{2+}/Cu$^+$ couple pH between 5.5 and 8.7 as shown in Fig. 3 of ref. 6. It is reasonable to assume that in the reduced form of the protein the imidazolium anion becomes dissociated from one of the metal ions and binds a proton. Below pH 5.5 E_m becomes substantially independent of pH suggesting that the group responsible for the pH dependence of E_m above pH 5.5 binds a proton in the absence of Cu^{2+} reduction below pH 5.5. In the context of this assumption, the spectral manifestations of the low pH transition can be interpreted in terms of the equilibrium

$$Zn^{2+}-N\ominus N-Cu^{2+} \xrightleftharpoons{H^+} \begin{array}{l} Zn^{2+}-N \diagup NH \quad Cu^{2+} \\ \text{or} \\ Zn^{2+}-HN \diagup N-Cu^{2+} \end{array}$$

where the imidazolium anion becomes protonated with concomitant dissociation from one of the metal ions. On the basis of the present observations it is not possible to distinguish between the two cases, but a simple consideration of the relative affinity constants suggests that the imidazole would remain coordinated to Cu^{2+} rather than Zn^{2+} [25].

It is useful to compare the low pH optical spectrum with that of a derivative

of the protein in which the Zn^{2+} binding site is unoccupied but Cu^{2+} is bound in the Cu^{2+} site, 2 Cu^{2+}-protein [26, 27]. It has been shown that the optical spectrum of this species lacks the absorption band near 450 nm, observed in the native spectrum, just as does the low pH form of the native protein. The 450 nm absorption can be restored to the 2 Cu^{2+} derivative when Zn^{2+}, Hg^{2+}, Cd^{2+} [26] or Cu^{2+} [27] binds to the Zn^{2+} site, and this suggests that 450 nm absorption depends on the presence of the metal bridging imidazolium anion. The loss of this band in the native protein at low pH suggests but does not prove that the bridging imidazolium anion becomes protonated at low pH.

The low pH transition does not consist only of the above-mentioned ionization. The PMR spectra (Fig. 6) indicate minor structural alterations associated with both aliphatic and aromatic residues. Further, the equilibrium must be strongly linked to other ionizations as evidenced by the qualitative observation that the binding of a substantial number of protons occurs subsequent to a slower process which may be a general conformational change resulting from the proton-induced structural change at the active center. A conformational modification affecting the frictional coefficient of the molecule could account for the small decrease in the sedimentation coefficient; the decrease, notably, is only slightly larger than that obtained on removing the metal ions (Fig. 5).

Apoprotein

Near neutral pH the apoprotein exists as a folded structure in which the two subunits are clearly associated as evidenced by the sedimentation coefficient. The PMR spectrum of "native" apoprotein indicates a somewhat different three-dimensional structure than that of holoprotein, and the metal ions clearly play an important role in determining the protein's structure as has already been reported by Stokes et al. [28]. The effect of bound metals on the stability of the protein is particularly evident at low pH where the apoprotein is largely denatured, while this disruption of the compact globular structure is prevented by the bound metal ions. Previous studies have shown that metals enhance the thermal stability of the apoprotein near neutral pH [29] and its stability toward denaturing solvents [30].

At low pH the sedimentation coefficient has substantially decreased, from 2.56 S to 1.29 S. Assuming a random coil of 140 amino acids, the sedimentation coefficient in 6 M guanidine·HCl would be expected to be $S^\circ = 0.45$ S at zero protein concentration [31]. If we assume the same empirical function of S° for a random coil in guanidine·HCl holds in 0.1 M NaCl and correct for the difference in buoyant density, S° should be slightly greater than 0.8 S for the subunit of superoxide dismutase. On this basis the S_{eff} value obtained is consistent with substantial denaturation toward a random structure. The observed PMR spectrum supports this contention but some three-dimensional structure appears to remain as evidenced by the broad underlying resonance envelopes remaining even at p^2H 2.9. Thus, a small amount of "core" structure probably remains.

Some comments on the mechanism of reconstitution

The resolution of metals from protein generally involves treatment of the holoprotein with EDTA at pH 3.8 [7], and this procedure, which yields the starting material for reconstitution, has been shown to effectively remove 90–99 % of both

metals [4, 26, 32, 33]. Reconstitution to native protein either partially or fully has been effected over a range of conditions. Weser et al. [33] were able to obtain a partial yield of native protein in a mixture, judging from published EPR spectra, of non-native Cu^{2+}-protein complexes at pH 7.2, 20 °C, 10 min with stoichiometric ratios of metal to protein. We found previously [3] that dialysis of apoprotein against a dilute metal solution containing a 10-fold excess of metals led to about a 70% yield of native protein having no non-native Cu^{2+}-protein contaminants. The time required was approx. 30 h and the optimum pH was near 5. Beem et al. [26] have recently described a procedure which involves slow addition of 2 molar equivalents of Cu^{2+} to apoprotein maintained at pH 3.8 followed by direct addition of 2 molar equivalents of Zn^{2+}. This procedure returns native protein in very high yield.

Since a substantial fraction of apoprotein appears to exist in a monomeric, random coil state at pH 3.8 the reconstitution process requires a return of this to a folded structure. The results of Beem et al. [26] suggest that the highest yield of reconstituted protein can be obtained if the refolding is effected in the presence of Cu^{2+} at a pH where apoprotein can dynamically unfold and refold, the Cu^{2+} stabilizing the so-called 2 Cu^{2+}-protein [26, 27] which readily binds Zn^{2+} to form native protein [3, 26]. The lower yields obtained when reconstitution was effected at higher pH values can be explained by a refolding of apoprotein to a globular structure which slowly and only partly rearranges to the native structure in the presence of Zn^{2+} and Cu^{2+}.

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