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^{35}Cl and ^{1}H NMR Study of Anion Binding to Reduced Bovine Copper-Zinc Superoxide Dismutase

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Binding of chloride to reduced bovine copper-zinc superoxide dismutase (Cu_2Zn_2SOD) and chemically modified derivatives was monitored by the line width at half-height of the Cl^- resonance as measured by ^{35}Cl nuclear magnetic resonance (NMR) spectroscopy. Reduced arginine-modified and reduced lysine-modified Cu_2Zn_2SOD (at concentrations of 2.14×10^{-4} M) caused less broadening of the Cl^- resonance line width of 0.1 M NaCl solutions than did reduced native protein when measured under the same conditions; Cl^- broadening with all protein derivatives decreased drastically in the presence of 0.05 M phosphate. The C-H and N-H proton resonances of histidyl imidazoles of reduced native and reduced lysine-modified Cu_2Zn_2SOD were shifted by addition of Cl^- (with apparent affinity constants of 12 and ~ 2 M^{-1}, respectively) whereas this anion had less effect in the ^1H NMR spectrum of reduced arginine-modified Cu_2Zn_2SOD (affinity constant <2 M^{-1}) under the same conditions. Phosphate caused relatively smaller changes on the ^1H NMR resonances of all reduced protein derivatives. The competition measured by ^1H NMR spectroscopy between chloride and phosphate for anion binding sites in the neighborhood of the Cu^I ion was much less than that for nonspecific Cl^- binding monitored by ^{35}Cl NMR spectroscopy. We conclude from these experiments that, in addition to the weak anion binding at or near the Cu^I ion, Arg-141, Lys-120, and Lys-134 serve as major anion binding sites in the reduced native protein. Thus, electrostatic interactions between the positively charged arginine and lysine side chains and the substrate, superoxide anion, are presumably present in the reduced state of native Cu_2Zn_2SOD and may play a role in its mechanism of superoxide disproportionation.

Introduction

Bovine erythrocyte copper-zinc superoxide dismutase (Cu_2Zn_2SOD) has two identical subunits in its oxidized form, each subunit containing one Cu^{II} ion and one Zn^{II} ion. This protein is an extremely efficient catalyst of superoxide dismutation ($2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$), and it has been proposed that this activity is its primary physiological function in vivo.[2] The reactive site is the Cu ion, which undergoes alternate reduction and oxidation during the catalytic cycle. A great deal of structural information is available for this protein in that both its amino acid sequence[3] and X-ray crystal structure (oxidized form only) at 2-Å resolution[4] are known. The Cu^{II} ion is coordinated to four imidazole nitrogens from histidines-44, -46, -61, and -118 and to a water molecule, making the overall geometry five-coordinate.[4] The Zn^{II} ion is coordinated to imidazoles from histidines-61, -69, and -78 and the carboxylate group from aspartate-81 in an approximately tetrahedral geometry. The structure of the active site of bovine Cu_2Zn_2SOD in its oxidized form is unique among structurally characterized metalloproteins in that the imidazole ring of histidine-61 is deprotonated and bridges the Cu and Zn binding sites. There are eight histidine residues per subunit. The two remaining ones are histidine-19, which is known from the X-ray studies to be accessible to solvent, and histidine-41, which is less accessible since it is buried in the β-barrel structure of the protein.

Bovine Cu_2Zn_2SOD in its oxidized form has a high affinity for anions, and it is clear from spectral changes observed upon anion binding that the major anion binding site is the Cu^{II} ion.[5] Phosphate, unlike other anions, does not bind to the Cu^{II} ion.[6,7] However, phosphate interacts with the protein via the positively charged side chains of Arg-141, Lys-120, and Lys-134,[6,7] which are located in the vicinity of the active site, 5, 12, and 13 Å, respectively, away from the Cu^{II} ion.[4]

In the case of the reduced protein, i.e. $Cu^I_2Zn^{II}_2SOD$, there is considerable evidence indicating that anions interact with the protein,[8-10] but the exact nature of the anion binding site is still unknown.[5] Fee and Ward[8] concluded from ^{35}Cl NMR studies carried out with reduced bovine Cu_2Zn_2SOD and apoprotein that an open coordination site existed on one of the two metal ions in the reduced protein, the most likely site being the Cu^I ion. The binding of halides to reduced yeast Cu_2Zn_2SOD was also studied by high-field ^1H NMR spectroscopy.[9] The yeast protein contains six histidines per subunit (as opposed to eight for bovine Cu_2Zn_2SOD) and, since all six are in the metal binding sites,[10] the yeast protein is particularly well suited for ^1H NMR studies of the metal binding region. Five resonances were assigned to C-2 protons of histidyl imidazoles in the reduced protein, and three of these resonances were observed to shift upon addition of halide ions. Although these observations are consistent with coordination of halide ions to the Cu^I ion in reduced yeast Cu_2Zn_2SOD,[9] they can also be interpreted as binding of halide ions to a protein side chain close to the metal binding region.[5] Rigo et al.[11] reported that the binding constants for anions calculated from measurements of the effect of anions on T_1 relaxation times of aqueous solutions of oxidized bovine Cu_2Zn_2SOD were generally lower than those measured from the degree of inhibition of SOD activity. Since the former method involved binding only to the oxidized form whereas the latter involves both the oxidized and reduced forms, these authors suggested higher binding constants for anions to the reduced form than to the oxidized form of the protein.

In this paper, we reevaluate these data in light of the present understanding of electrostatic interactions in bovine Cu_2Zn_2SOD. It has been shown from the SOD activity and anion affinity of proteins chemically modified at Arg-141 with phenylglyoxal[12,13] or at Lys-120 and Lys-134 with succinic anhydride, acetic anhydride, or cyanate[14-17] that these residues are important anion

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binding sites in the oxidized protein.[6,7,13,15] We report here ^{35}Cl NMR and high-resolution ^1H NMR studies on the binding of Cl^- to reduced native and reduced chemically modified proteins. Interaction of phosphate to the above-mentioned forms of SOD were also investigated by high-resolution ^1H NMR spectroscopy. These studies support the conclusion that in reduced bovine Cu_2Zn_2SOD Arg-141, Lys-120, and Lys-134 serve as anion binding sites in addition to anion binding sites in the immediate neighborhood of the Cu^I ion.

Experimental Section

Bovine liver Cu_2Zn_2SOD was purchased as lyophilized powder from Diagnostic Data, Inc. (Mountain View, CA). Phenylglyoxal, succinic anhydride, sodium dithionite, HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], xanthine, xanthine oxidase (grade I), and horse heart cytochrome *c* (type III) were supplied by Sigma; potassium phosphate dibasic trihydrate and sodium chloride were from Mallinckrodt, Inc.; zinc nitrate was from J. T. Baker Chemical Co.; and deuterium oxide (99.8% and 99.95%) was supplied by Cambridge Isotope Laboratories and Chemical Dynamics Corp., respectively. All were used as received.

Bovine Cu_2Zn_2SOD was chemically modified at Arg-141 with phenylglyoxal and purified by the method of Malinowski and Fridovich.[12] Succinylation of lysine residues of bovine Cu_2Zn_2SOD was carried out according to Marmocchi et al.[14] The purified arginine- and lysine-modified proteins were dialyzed against distilled water and lyophilized. The purity of the modified protein derivatives was confirmed by ^1H NMR spectra (Figures 3–5) that showed complete disappearance of the characteristic signals of the reduced native protein. Protein concentrations were determined by Lowry assay[18] or weight of lyophilized powder to be dissolved. SOD activity of arginine- and lysine-modified proteins was approximately 15% and 20% of that of native protein as measured by the xanthine oxidase–cytochrome *c* assay system.[19] Reduced proteins were obtained by addition of small amounts of solid sodium dithionite until the color of the protein was bleached. Apo-SOD was prepared according to Lippard et al.[20] and stored in 50 mM acetate buffer, pH 5.5. Samples of apo-SOD prepared for NMR work were obtained by dialyzing extensively against 0.25 M HEPES buffer, pH 7.3, or 0.05 M phosphate buffer, pH 7.4. The concentration of the resulting apo-SOD solutions was determined by the Lowry method.[18] Samples of E_2Zn_2-SOD[21] for NMR work were obtained by addition of 2 equiv of Zn^{II} per dimer to apo-SOD solutions at room temperature. Metal analysis of the E_2Zn_2SOD preparations by atomic absorption photometry (Perkin-Elmer Model 603 instrument) showed that the Zn^{II} ion concentration was the same as that of the native, while the metal content of the apo-SOD preparations was less than 5%. As shown previously,[20] addition of 2 equiv of Zn^{II} per dimer to apo-SOD resulted in full incorporation of Zn^{II} at the zinc site of the protein.

^{35}Cl NMR measurements (UCLA) were made at 8.73 MHz on a JEOL FX90Q NMR spectrometer equipped with a multinuclear probe. These measurements were carried out at both the ambient probe temperature (32 °C) or at higher temperatures with a variable-temperature unit. The samples were contained in 10-mm NMR spinning tubes. ^{35}Cl NMR spectra were measured with 2000-Hz spectral width by using 4K data points. Prior to Fourier transformation, exponential filtering was applied to the FID (free induction decay) to enhance signal-to-noise ratio and the resultant line broadening (4.0 Hz) was subtracted from the observed line widths.

The ^1H NMR spectra were recorded on a Bruker AM500 NMR spectrometer (UCLA) at 500 MHz and on a Varian VXR-400 NMR spectrometer (Northwestern University) at 400 MHz. Preliminary ^1H NMR spectra were also obtained at 500 MHz on a Bruker WM500 NMR spectrometer at the Southern California Regional NMR Facility at California Institute of Technology, Pasadena, CA. Typical spectra consisted of about 2000 scans with 32K data points over a wide enough bandwidth to cover all the protein signals. Line broadening of 2 Hz was employed for the spectra taken in H_2O solutions. Resolution enhancement of the spectra obtained in D_2O solutions was accomplished by applying convolution difference[9] to the FIDs using approximately 15-Hz line broadening. Temperature was maintained at 25 °C. DSS (4,4-dimethyl-4-silapentane-1-sulfonate) was used as an internal reference. Solutions for ^1H NMR spectroscopy were prepared by dissolving the protein in D_2O (99.8%) or in neat H_2O at room temperature. Insoluble matter, if any, was removed by Millipore filtration. Selective excitation of the reduced protein derivatives in water was recorded unlocked by using the 1-3-3-1 (UCLA) or 1-1 (Northwestern University) hard pulse sequences.[22] The maximum excitation was centered around the signals of interest, while the null was set on H_2O.

The pH of the solutions was measured directly in the NMR tube with an Orion pH meter. A semimicro Polymark combination electrode or a MI 412 microcombination pH probe was used for 10- or 5-mm NMR tubes, respectively. NaOD and DCl solutions (Aldrich) were used for pH adjustments in the D_2O solutions. Thus, pH* values quoted for the D_2O solutions are not corrected for the deuterium isotope effect.

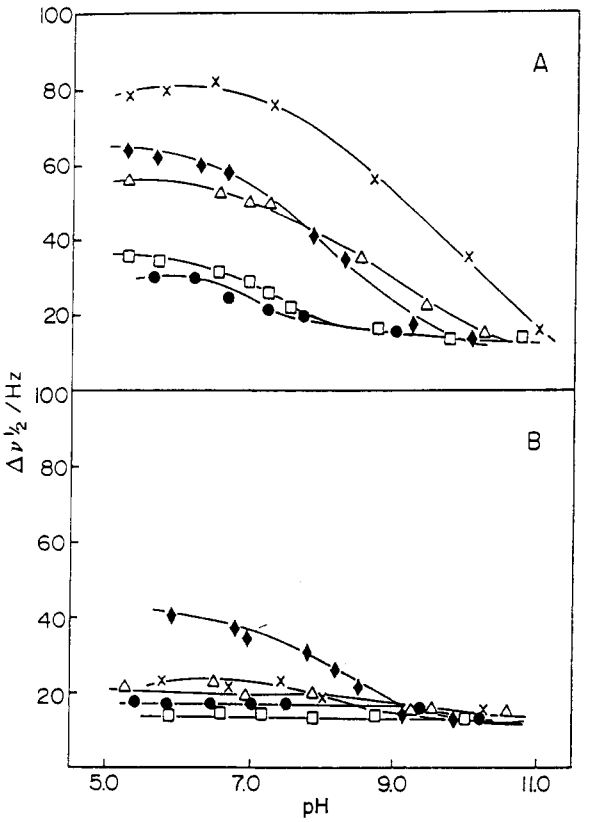

Figure 1. (A) pH dependence of the $^{35}Cl^-$ NMR resonance of 0.1 M sodium chloride in the presence of 2.14×10^{-4} M reduced native SOD (×), reduced arginine-modified SOD (△), reduced lysine-modified SOD (□), Zn-only SOD (♦), and apo-SOD (●) in 0.25 M HEPES. (B) Same experimental conditions as in (A) with the exception that the buffer solution is 0.05 M phosphate.

Results

Binding of Cl^- to reduced native bovine Cu_2Zn_2SOD and protein derivatives was monitored by the line width at half-height of the Cl^- resonance as measured by ^{35}Cl NMR spectroscopy. Figure 1A shows that the Cl^- line width in solutions containing 0.1 M NaCl and reduced native Cu_2Zn_2SOD (2.1×10^{-4} M) in 0.25 M HEPES in the pH range 5.0–7.0 is approximately 80 Hz. Similar experiments carried out with equivalent concentrations of NaCl and reduced arginine- or lysine-modified SOD, in the same pH range, gave Cl^- line widths of approximately 55 and 35 Hz, respectively.

Different buffer concentrations (0.25 M HEPES and 0.05 M phosphate) were used in experiments in the absence and presence of phosphate (Figure 1A,B) so that a similar contribution toward

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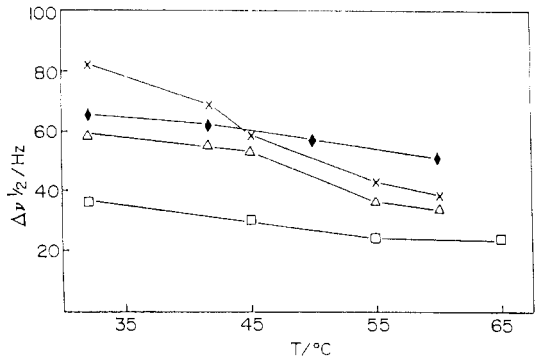

Figure 2. Temperature dependence of the $^{35}Cl^-$ NMR resonance of 0.1 M sodium chloride in the presence of 2.14×10^{-4} M reduced native SOD (×), reduced arginine-modified SOD (△), reduced lysine-modified SOD (□), and Zn-only SOD (♦) in 0.25 M HEPES buffer, pH 5.30. The apoprotein was not studied by this method, as this protein derivative is known to denature in this temperature range.[28]

ionic strength was obtained. It was important to ensure that ionic strength remained constant during anion binding studies of this protein, as it had been previously shown that the SOD activity and anion affinity of bovine Cu_2Zn_2SOD were highly dependent on ionic strength.[6,16] We showed previously that phosphate interacted with oxidized native bovine Cu_2Zn_2SOD via the positively charged side chains of Arg-141, Lys-120, and Lys-134.[6,7] Figure 1B indicates that the Cl^- line width in the presence of reduced native or reduced chemically modified proteins in 0.05 M phosphate buffer is almost as sharp as that of free Cl^- in solution and virtually independent of pH. We interpret the sharpness of the Cl^- resonance as being due to competition between phosphate and chloride for the same binding sites. In HEPES solutions and at high pH, the Cl^- resonance line widths become sharper, suggesting that OH^- ions may be competing effectively with Cl^- for the same binding sites. Alternatively, deprotonation of lysine residues would lead to an increased overall negative charge on the protein, which could be responsible for the decreased anion affinity at alkaline pH.[23,24] At high pH and in the modified proteins, the Cl^- affinity for the proteins drops to zero. It is unlikely that the overall charge on the protein determines the Cl^- affinity for the reduced proteins, since it is known that the dependence of anion binding and superoxide activity of Cu_2Zn_2SOD on ionic strength[6,16] indicates that specific charged residues, rather than the overall charge on the protein, control the electrostatic interactions between anions, including the superoxide substrate, and Cu_2Zn_2SOD.

The decrease in Cl^- line width observed with increasing temperature for reduced native SOD, reduced arginine-modified SOD, reduced lysine-modified SOD, and E_2Zn_2SOD shown in Figure 2 is indicative of fast exchange between free chloride in solution and protein-bound chloride.[8,25-27] Moreover, the $^{35}Cl^-$ chemical shift of a 0.1 M NaCl solution is the same in the presence or absence of 2.14×10^{-4} M reduced native or chemically modified protein. Exchange broadening cannot therefore account for the larger Cl^- line broadening observed in the case of chloride solutions of reduced native SOD.

Decreased chloride line widths were also observed in sample solutions containing Zn-only SOD (E_2Zn_2SOD) and apo-SOD relative to solutions of reduced native SOD (see Figure 1A). These results indicate a lowering of the affinity of the protein for chloride when copper is removed. In the case of the apoprotein, phosphate apparently displaces chloride, but in the case of E_2Zn_2SOD, the chloride line width is substantially broadened relative to free chloride in the presence of phosphate.

Table I. ^1H NMR C-H Chemical Shifts (δ, ppm from DSS) of Reduced Derivatives of Bovine $Cu^I_2Zn^{II}_2SOD$[a-c]

form of SOD	resonance no. 1	2	3	4	5	6
native	8.63	8.60	8.49	8.44	8.09	7.73
native + Cl^-	8.64	8.70	8.49	8.62	8.30	7.73
Lys-modified	8.52	8.50	8.31	8.23	7.81	7.58
Lys-modified + Cl^-	8.55	8.55	8.34	8.45	8.13	7.63
Arg-modified	8.61	8.57	8.48	8.48	8.08	*d*
Arg-modified + Cl^-	8.64	8.69	8.49	8.64	8.26	*d*

[a] $[SOD]_0$ = 0.5 mM, pH* 6.5, $[Cl^-]$ = 0.57 M except for Arg-modified samples where $[Cl^-]$ = 0.89 M. [b] Resonances 1-6 are due to C-2 histidyl imidazole protons that have been assigned for the reduced native protein.[29,35,36] Resonances 2 and 4 are due to Cu ligands whereas 3 and 6 are due to Zn ligands. Resonance 1 was assigned to His-41, and 5, to His-19. His-41 and His-19 are not in the metal binding region. However, only His-19 is accessible to solvent and this is why the chemical shift of resonance 5 is sensitive to small pH changes.[35] [c] The signal assignments for the reduced native and reduced lysine-modified derivatives were made by tracing the individual resonances during Cl^- titration. A Cl^- titration was not performed for reduced arginine-modified SOD due to the small affinity constant (see text). The signal assignment for this derivative was made by analogy to the other reduced proteins. [d] Resonance 6 of reduced arginine-modified SOD is buried under the resonance envelope of phenylglyoxal and could not be assigned unambiguously.

Table II. ^1H NMR N-H Chemical Shifts (δ, ppm from DSS) of Reduced Derivatives of Bovine $Cu^I_2Zn^{II}_2SOD$[a-c]

form of SOD	resonance no. 7	8	9	10	10′	11	11′
native	15.31	13.96	13.49	12.82	12.56	12.48	12.20
native + Cl^-	15.35	13.96	13.46	12.86	12.56	12.49	12.21
Lys-modified	15.30	13.89	13.39	12.84	12.57	12.48	12.20
Lys-modified + Cl^-	15.35	13.96	13.49	12.83	12.59	12.50	12.22
Arg-modified	15.38	13.91	13.36	12.83	*d*	12.55	12.29
Arg-modified + Cl^-	15.40	13.96	13.42	12.83	*d*	12.57	12.32

[a] $[SOD]_0$ = 0.5 mM, $[Cl^-]$ = 0.57 M except for Arg-modified samples where $[Cl^-]$ = 0.89 M. [b] Resonances 7-11 are due to N-1 and N-3 protons of histidyl imidazoles that have been assigned for the reduced native protein.[30] Resonances 7 and 9 have been tentatively assigned to metal-bond N-3 protons of either His-44 or His-69, while 8 and 10 have been assigned to N-3 and N-1 protons of His-41, respectively.[30] The assignment for resonance 11 is still unknown.[30] [c] The signal assignments for the reduced native and reduced lysine-modified derivatives were made by tracing the individual resonances during Cl^- titration. A Cl^- titration was not performed for reduced arginine-modified SOD due to the small affinity constant (see text). The signal assignment for this derivative was made by analogy to the other reduced proteins. [d] Resonance 10′ of reduced arginine-modified SOD is not resolved.

Figure 3 shows the effect of Cl^- on the C-H proton resonances of histidyl imidazoles for reduced native and reduced lysine-modified SOD in D_2O. Addition of Cl^- changed the chemical shifts of active-site C-H histidyl resonances for both reduced native and reduced lysine-modified SOD. Figure 4 indicates that the changes in the N-H signals from reduced native SOD in H_2O were very pronounced in that two new signals at 12.49 and 12.21 ppm were resolved upon addition of Cl^-. The appearance of two new N-H resonances upon addition of Cl^- to reduced native protein is probably due to decreased lability of N-H protons. By contrast, smaller changes were observed in the ^1H NMR spectra of reduced arginine-modified SOD in D_2O and H_2O (Figure 5 and Table I) upon addition of high concentrations of Cl^-. A Cl^- concentration dependence study of C-H chemical shifts yielded apparent Cl^- affinity constants of approximately 12 and ≤ 2 M^{-1}, respectively, for reduced native and reduced lysine-modified proteins. Detectable changes in the ^1H NMR spectrum of reduced arginine-modified Cu_2Zn_2SOD occurred at much higher Cl^- concentrations, suggesting an affinity constant of <2 M^{-1} for this protein derivative.

Tables I and II show the effect of Cl^- on the chemical shifts of C-H and N-H histidyl imidazole proton resonances. Reso-

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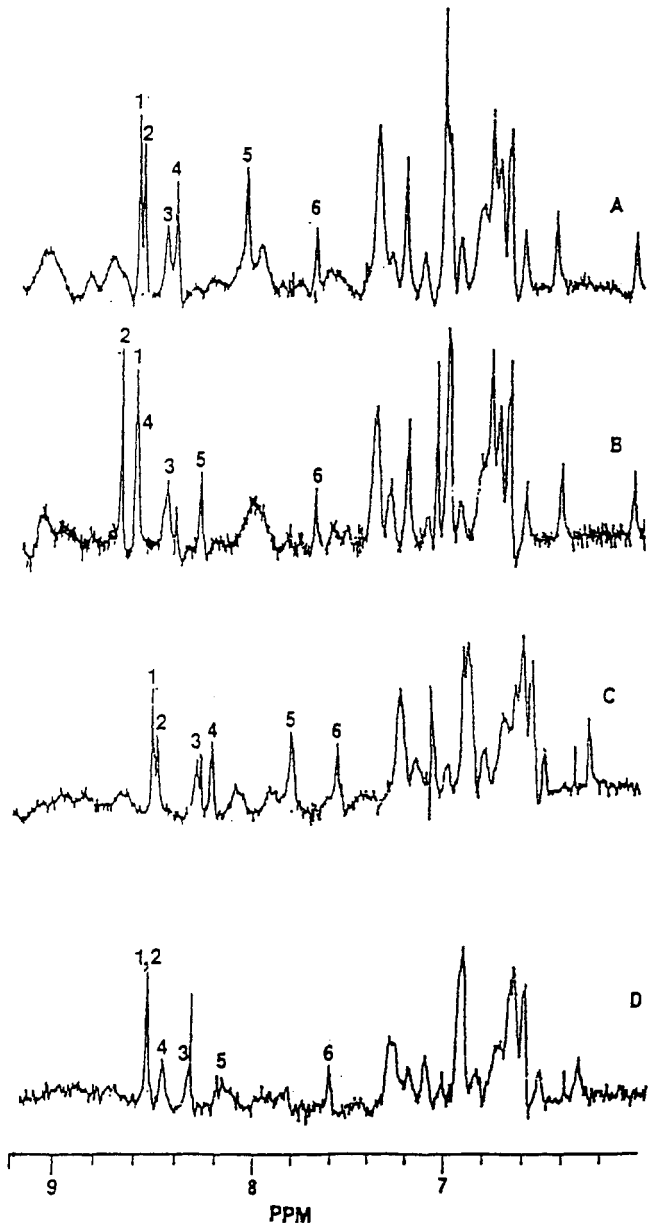

Figure 3. ^1H NMR spectra (500 MHz; 25 °C) of (A) reduced native SOD, (B) reduced native SOD + 0.57 M Cl^-, (C) reduced lysine-modified SOD, and (D) reduced lysine-modified SOD + 0.89 M Cl^-. All protein samples at 0.5 mM concentration were prepared in D_2O and were unbuffered (pH* was approximately 6.5). For assignments of resonances 1–6, see Table I.

nances 2 and 4 from the reduced native protein were previously assigned to Cu ligands.[29] The assignments of N–H proton resonances for the reduced native protein, based on a previous study,[30] are given on Table II. Both the C–H and N–H proton resonances from active-site histidines of reduced native and of reduced modified proteins were shifted upon Cl^- addition. Resonances 2 and 4, assigned to histidines in the copper site, were most affected. However, the C–H and N–H resonances of reduced modified Cu_2Zn_2SOD, and in particular those from the arginine-modified derivative, were only shifted at much higher Cl^- concentrations, consistent with their lower affinities for chloride binding. ^1H NMR spectra of reduced native and reduced chemically modified protein samples in 0.15 M phosphate were also recorded (data not shown). In apparent contradiction to results described earlier where chloride line width measurements in the

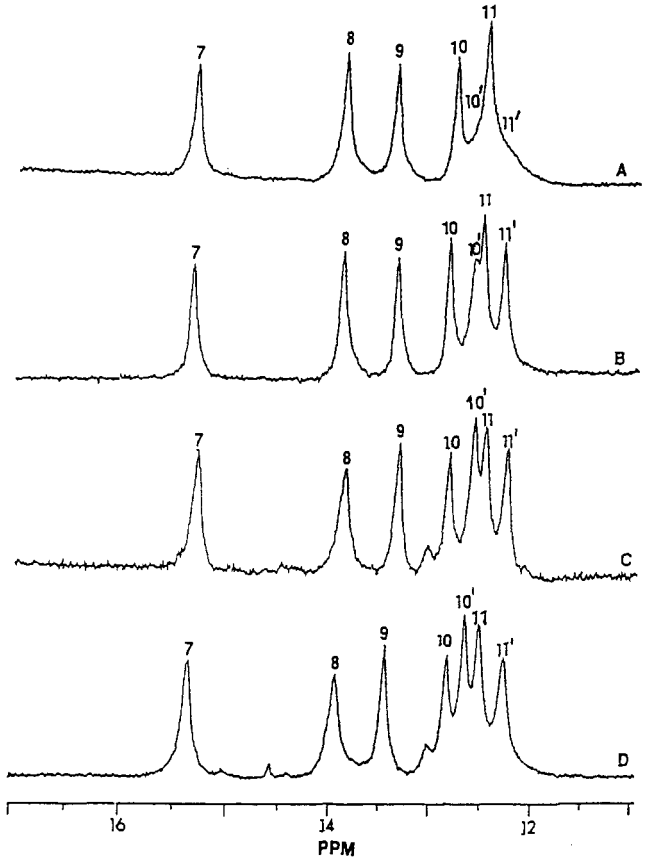

Figure 4. ^1H NMR spectra (500 MHz; 25 °C) of (A) reduced native SOD, (B) reduced native SOD + 0.57 M Cl^-, (C) reduced lysine-modified SOD, and (D) reduced lysine-modified SOD + 0.70 M Cl^-. The experimental conditions were the same as for Figure 3 except that the protein samples were dissolved in H_2O, as opposed to D_2O, enabling the detection of N–H histidyl imidazole resonances. For assignments of resonances 7–11, see Table II.

presence of 0.05 M phosphate indicated that phosphate displaced chloride from the protein (vide supra), C–H proton resonances of histidyl imidazoles of reduced native and reduced lysine-modified Cu_2Zn_2SOD were shifted less by phosphate than by chloride upon addition of similar anion concentrations. C–H resonances 2 and 4 were shifted by 0.05 ppm or less upon addition of phosphate, while shifts of approximately 0.20 ppm were induced by the addition of a similar concentration of chloride. When both chloride and phosphate were added to the reduced native protein, the C–H resonances were shifted by approximately 0.2 ppm, indicating that phosphate does not compete effectively with Cl^- for anion binding sites in the neighborhood of the metal. The weak competition between Cl^- and P_i measured by ^1H NMR spectroscopy is in contrast with the strong anion competition monitored by ^{35}Cl NMR spectroscopy (Figure 1). Similarly to what was observed with Cl^- binding, phosphate had a smaller effect on the ^1H NMR spectra of reduced arginine-modified Cu_2Zn_2SOD relative to the other reduced protein derivatives.

Chemical modification of native bovine Cu_2Zn_2SOD at Arg-141 with phenylglyoxal or at Lys-120 and Lys-134 with succinic anhydride resulted in changes at the active site of both reduced chemically modified proteins as manifested by shifts of the C–H and N–H proton resonances (cf. Figures 3A, 3C, and 5A and Figures 4A, 4C, and 5C, respectively). We had noted previously that chemical modification of Arg-141 with phenylglyoxal shifted the environment of the Cu^{II} ion to a more nearly axial coordination geometry.[13] We can now conclude that the chemical modification of Arg-141 with phenylglyoxal affects the active-site structure of both the oxidized and reduced forms of the protein. Although the chemical modification of lysine residues with succinic anhydride apparently does not affect the active-site structure of the oxidized form,[14,15] our ^1H NMR results indicate that the active-site structure of the reduced protein does change upon succinylation.

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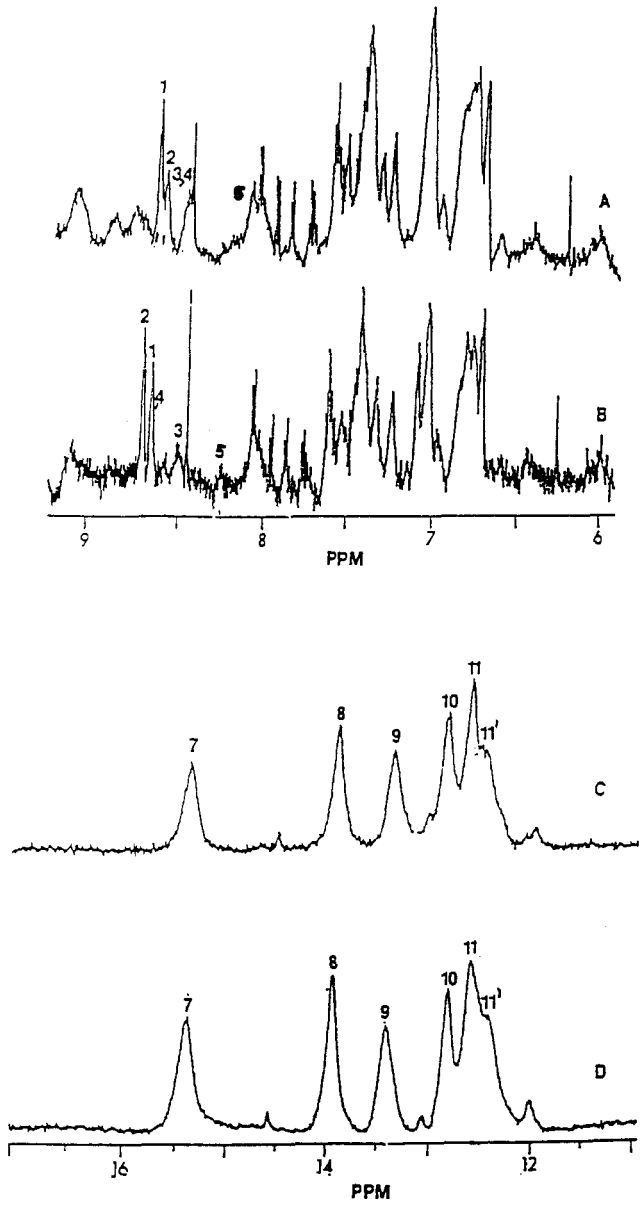

Figure 5. ^1H NMR spectra (500 MHz; 25 °C) of (A) reduced arginine-modified SOD (0.5 mM) in D_2O, (B) sample A + 0.89 M Cl^-, (C) reduced arginine-modified SOD (0.5 mM) in H_2O, and (D) sample C + 0.89 M Cl^-.

Discussion

^{35}Cl NMR has been applied extensively to a large number of water-soluble protein systems to monitor Cl^- interactions with proteins.[26,27] Fee and Ward[8] first showed that ^{35}Cl NMR spectroscopy could be used to study the binding of Cl^- to reduced bovine Cu_2Zn_2SOD. The physical basis of this experimental approach is the large difference in the line widths of bound and free chloride; the line width of protein-bound chloride is typically more than 10^4 times that of free chloride in solution.[31] When chloride is in fast exchange between protein binding sites and solution and no chemical shift change occurs upon its binding to proteins, the observed line width of the Cl^- resonance is a weighted average of protein-bound and free chloride.[26,27] Because of the relatively small concentration of bound chloride (in our experiments, the [SOD]/[total Cl^-] ratio is of the order of 10^{-3}), the observed ^{35}Cl NMR resonance is still reasonably sharp and its line width is not far from that of free chloride in solution.

Figure 1A indicates that the Cl^- resonance line width of 0.1 M NaCl solutions containing 2.14×10^{-4} M reduced native bovine Cu_2Zn_2SOD is approximately 1.5 and 2.3 times greater than the Cl^- line width measured, under the same concentration conditions, for solutions containing reduced arginine-modified or reduced lysine-modified protein. This decrease in the Cl^- resonance line width indicates that the reduced chemically modified proteins have lower affinities for chloride binding in the order reduced native > reduced arginine-modified > reduced lysine-modified. The Cl^- association constants for reduced native and reduced modified proteins obtained from Cl^- titrations of the ^1H NMR spectra (see Results) confirmed the reduced anion affinities for the modified derivatives, but in this case the apparent order is reduced native > reduced lysine-modified > reduced arginine-modified. The observation that phosphate, which is known to interact with bovine Cu_2Zn_2SOD in the oxidized[6,7] and reduced states via the arginine and lysine residues and not through the metal center, drastically decreases the Cl^- resonance line width in solutions containing either reduced native or reduced chemically modified proteins to approximately the same value (see Figure 1B) suggests the possibility that the bulk of chloride binding is to the arginine and lysine residues. Moreover, phosphate had a smaller effect than chloride on the histidyl proton C–H resonances, and the weaker competition between the two anions as measured by ^1H NMR spectroscopy, relative to that measured by Cl^- line broadening, indicates only weak anion binding in the immediate neighborhood of the Cu^I ion.

Fee and Ward[8] also studied the binding of Cl^- to apo-SOD by ^{35}Cl NMR spectroscopy and found that the line width of the Cl^- resonance in the presence of apoprotein solutions was approximately 2.5 times less than that observed in the presence of reduced native Cu_2Zn_2SOD and that addition of excess CN^- to the latter lowered the Cl^- line width to a value typical of Cl^- solutions in the presence of apo-SOD. Those authors interpreted their results as being due to Cl^- binding to the Cu^I ion and suggested that the effect of CN^- was to bind to Cu^I, thus displacing Cl^- from the coordination position. Evidence for interaction of CN^- with oxidized bovine Cu_2Zn_2SOD comes from an X-ray absorption study that showed that addition of CN^- to the oxidized protein resulted in changes at the Cu binding site.[32] Unfortunately, there is no X-ray absorption edge data on halide binding to reduced SOD. Thus, the Cl^- broadening data do not rule out the possibility of Cl^- binding to a side chain on the protein close to the Cu^I ion from which it may be displaced by CN^- binding to Cu^I or directly to the side chain. There are precedents for this type of interaction between Cl^- and arginine side chains. For instance, it was reported for band 3 protein, a membrane system present in red blood cells whose function is to exchange Cl^- for HCO_3^- ions, that Cl^- competes with the chemical modification of an arginine residue with phenylglyoxal.[33] Another precedent for Cl^- binding to proteins via amino acid side chains has been found in hemoglobin.[34]

We also studied the interaction of Cl^- with apo-SOD and E_2Zn_2SOD in the presence and absence of phosphate and found that the Cl^- line width in the presence of apo-SOD was sharper than that in the presence of E_2Zn_2SOD, which in turn was sharper than the Cl^- line width observed in the presence of reduced native Cu_2Zn_2SOD. We also observed that the presence of phosphate reduced the Cl^- affinities of both apo-SOD and E_2Zn_2SOD but that removal of chloride from E_2Zn_2SOD was incomplete. We conclude from this observation that the mode of chloride binding is different in E_2Zn_2SOD. It seems likely to us that the histidyl imidazoles of the empty copper site when protonated would very likely provide a local region of positive charge that might bind chloride. It was shown previously that the Zn^{II} ion plays a structural role in bovine Cu_2Zn_2SOD from the observation that addition of 2 equiv of Zn^{II} ion per subunit to apo-SOD gives a ^1H NMR spectrum which is very similar to that of reduced native bovine $Cu^I_2Zn^{II}_2SOD$.[20,29,35] The same ^1H NMR studies showed

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that the structure of apo-SOD is significantly different from that of E_2Zn_2SOD. It is likely therefore that this conformational change is bringing the copper site histidines into close proximity. Whatever the cause for the reduced Cl^- affinities of apo-SOD and E_2Zn_2SOD, it is apparent from our ^{35}Cl NMR results that it is not possible to decide for certain from Cl^- binding studies on these protein derivatives whether or not the Cu^I ion itself or anion binding sites in the immediate neighborhood of the Cu^I ion contribute toward anion binding in reduced native bovine Cu_2Zn_2-SOD, in addition to the arginine and lysine residues.

To investigate further the nature of chloride binding to reduced native and reduced chemically modified proteins, we carried out a study using ^1H NMR spectroscopy. Assignments of C–H proton resonances of histidyl imidazoles for reduced native yeast and bovine SOD were made previously by Cass et al.[9,29,36] Similar assignments have been made for the histidyl imidazole N–H resonances of reduced bovine Cu_2Zn_2SOD.[30] Three of the five resonances assigned to C-2 protons of histidyl imidazoles in reduced yeast Cu_2Zn_2SOD shifted upon addition of halide ions (including Cl^-), and these results were interpreted as being due to coordination of halide ions to the Cu^I ion. Figures 3–5 and Tables I and II indicate that Cl^- shifts the position of the C–H and N–H proton resonances of reduced native, reduced lysine-modified, and reduced arginine-modified SOD. However, there appears to be little pattern in the magnitude and direction of the chemical shifts. These results suggest that chloride binding to the protonated histidine-61 residue or to the N–H histidyl protons of the other copper ligands, which are known to participate in hydrogen bonding to other residues, may occur. Cl^- binding could substantially perturb the hydrogen bonding interactions around the Cu^I site, leading to the observed small chemical shift changes. This weak anion binding in the immediate neighborhood of the Cu^I ion may be responsible for the small shifts observed in the ^1H NMR experiments, in addition to direct chloride binding to the Cu^I ion. Since the reduced protein derivatives are diamagnetic, the small shifts observed by ^1H NMR spectroscopy could be also due to direct binding to Cu^I ion. A metal-based technique, rather than a ligand-based technique, will be required to differentiate between the binding at or near the Cu^I ion.

The possibility that chemical modification of Arg-141 is blocking the access of anions to the Cu^I ion cannot be ruled out. However, it is not likely, since it has been shown that oxidized arginine-modified Cu_2Zn_2SOD retains SOD activity and affinity for small anions, such as CN^-, N_3^-, and phosphate, although both of these are decreased to a similar extent upon chemical modification.[6,13,16] Recent ^1H NMR studies on oxidized human SOD mutants[37] showed that replacement of the arginine residue by amino acids of approximately the same size (lysine, isoleucine, and glutamate) resulted in a decrease in azide affinity. The mutant data indicate the importance of the arginine residue for anion binding and suggest that steric hindrance caused by phenylglyoxal modification is less significant than blockage of electrostatic interactions between the arginine side chain and anions. Although the Cl^- affinity constant shows the trend reduced native > reduced lysine modified > reduced arginine modified, the ^1H NMR spectrum of the reduced arginine modified protein does change upon addition of high Cl^- concentrations. Moreover, the small changes observed in the ^1H NMR spectra of all three types of reduced proteins during Cl^- titrations are indicative of common Cl^- binding site(s) in these proteins, which are most likely in the immediate neighborhood of Cu^I ion or on the Cu^I ion itself.

The ^1H NMR signals of the histidines in the metal binding site of the reduced proteins are not affected significantly by phosphate relative to chloride suggesting that phosphate may not bind to the reduced proteins at the Cu^I site. The fact that the apparent affinities for chloride binding follow different orders and that the degree of competition by phosphate is different in the two types of NMR experiments suggests that most of the chloride is bound to the protein at sites other than the Cu^I ion. Thus, we conclude that the bulk of chloride binding, which is responsible for the increased $^{35}Cl^-$ line width, occurs at sites not in the immediate neighborhood of the Cu^I ion and follow the binding order reduced native > reduced arginine-modified > reduced lysine-modified SOD, consistent with the magnitude of the change in the overall charge of the protein, and is completely displaced by phosphate. We conclude further that the small fraction of chloride binding which occurs at or near the Cu^I ion is responsible for the shifts in the ^1H NMR spectra, follows the binding order reduced native > reduced lysine-modified > reduced arginine-modified SOD, and is less readily displaced by phosphate. These results lead us to speculate that, in the case of reduced bovine Cu_2Zn_2SOD, there is probably only weak binding of Cl^- near or directly to the Cu^I ion. This is in agreement with the low affinity of N_3^- and CN^- for the Cu^I ion on the basis of redox equilibrium data between Cu^{II}/Cu^I in bovine Cu_2Zn_2SOD and $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ in the presence and absence of anions.[38] Steady-state kinetic studies of Cu_2Zn_2SOD[39] also indicate that N_3^- inhibition of enzyme activity involves a stronger interaction of this anion with the oxidized rather with the reduced form of the enzyme.

The results of the anion binding studies of reduced native bovine Cu_2Zn_2SOD described here have implications for the mechanism of superoxide disproportionation by this protein, since the substrate, O_2^-, is also an anion and is known to react with the enzyme in both oxidized and reduced forms.[39,40] Most of the characterization of bovine Cu_2Zn_2SOD has been on the oxidized form. The reasons for this are the air-sensitivity of the reduced form, the availability of the crystal structure of the oxidized form,[4] and the utility of electronic spectral techniques and ESR spectroscopy for characterization of Cu^{II} compared to the spectral silence of Cu^I. But the properties of the reduced protein are obviously just as important as those of the resting (oxidized) protein for an understanding of the mechanism of superoxide disproportionation. For instance, the reduction of the oxidized form by superoxide is a simple one-electron reduction of a metal by superoxide, a type of reaction that has been observed for superoxide in a number of low molecular weight metal complexes.[41] By contrast, the oxidation of the reduced protein by superoxide, which is known to occur with a rate at or near the diffusion limit,[39,40] is a type of reaction of superoxide that is not well understood and for which there are relatively few precedents.[41,42]

Reduced native Cu_2Zn_2SOD is believed to have a structure in which the imidazolate bridge present in the oxidized form has been cleaved and the imidazolate ring protonated on the side facing Cu. Observations in favor of this proposal are (1) the association of the reduction of native $Cu^{II}_2Zn^{II}_2SOD$ with the uptake of one proton,[43,44] (2) the similarity of the visible spectra of $Cu^I_2Co^{II}_2SOD$ and $E_2Co^{II}_2SOD$,[21,45–48] (3) the similarity of the ^{113}Cd NMR spectra of $Cu^I_2Cd^{II}_2SOD$ and $E_2Cd^{II}_2SOD$,[49,50] (4) the similarity of the ^1H NMR spectra of $Cu^I_2Co^{II}_2SOD$ and $E_2Co^{II}_2SOD$,[51,52]

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(5) the three isotopically shifted imidazole proton NH signals due to the coordinated histidines in the zinc site of $Cu^{I}_2Co^{II}_2SOD$[51,52] and $Cu^{I}_2Ni^{II}_2SOD$,[53] where one of the NH signals is due to the protonated formerly bridging His-61, and (6) the similarity of the X-ray absorption edge spectra of Zn^{II} in both oxidized and reduced Cu_2Zn_2SOD, suggesting that the imidazole ring of histidine-61 is coordinated to zinc in both species.[32,54]

The configuration of the Cu binding site in $Cu^{I}_2Zn^{II}_2SOD$ is still not known. In general, cuprous ion can form either linear, trigonal, or tetrahedral complexes.[55] It seems likely from ^1H NMR studies of reduced native protein that histidine-44, -46 and -118 remain coordinated to Cu^{I}.[20,30,36] It was previously proposed that there was a coordination site available on Cu^{I} in the reduced native protein and that this was thought to be the site that bound anions as well as the substrate, superoxide.[8,9,11] It was therefore proposed[8] that reduction of superoxide by $Cu^{I}_2Zn^{II}_2SOD$ requires binding of O_2^- to the Cu^{I} ion prior to electron transfer. This mechanism avoids the formation of the energetically unstable O_2^{2-} ion, since a proton can be readily donated by histidine-61 to the coordinated O_2^{2-} ion and the imidazolate bridge reformed. In this paper, we show evidence suggesting that Arg-141 plays a major role in enhancing chloride binding to the active-site region in reduced native Cu_2Zn_2SOD, and therefore we propose that electrostatic interactions between the substrate O_2^- and the positively charged side chain of Arg-141 are present not only in the oxidized form[6,7,13-16] but also in the reduced form of native Cu_2Zn_2SOD. This anion binding scheme for the reduced protein is in agreement with that proposed in two recent theoretical studies.[56,57] In these two studies, it was suggested that the close proximity of Arg-141 to the metal center leads to the formation of a stable superoxide–Cu^{II} intermediate that can oxidize another superoxide to oxygen with a simultaneous reduction of the Cu center. According to the mechanism proposed by Osman and Basch,[56] the resulting reduced form of the complex accepts a proton from Arg-141 and undergoes a charge distribution that leads to a new complex between Cu^{II} and a hydroperoxide anion. Addition of a second proton from the bridging histidine residue leads to release of the hydroperoxide anion in the form of hydrogen peroxide. Bertini's proposed mechanism[57] for superoxide reduction is different in that two protons (one from Arg-141 and the other from the bridging histidine residue) are added to the reduced form of the superoxide–enzyme complex causing an increase in the Cu–O distance prior to electron transfer. Both of these models of enzyme action suggest the importance of Arg-141 in the reduced form of native Cu_2Zn_2SOD. Our NMR data provide direct evidence that anion, and presumably substrate, binding to reduced Cu_2Zn_2SOD involves the Arg-141 residue.

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Registry No. SOD, 9054-89-1; Cl^-, 16887-00-6; PO_4^{3-}, 14265-44-2; Arg, 74-79-3; Lys, 56-87-1; Cu, 7440-50-8; His, 71-00-1.

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Activation of the BCO Unit in the Ketenylidene Analogue $(\mu\text{-H})_3Os_3(CO)_9(\mu_3\text{-BCO})$ by Electrophiles: Syntheses of Vinylidene and Alkyne Analogues

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The Lewis acids BX_3 (X = Cl, Br), BH_3, *B*-Cl-9-BBN, and $PhBCl_2$ react with $(\mu\text{-H})_3Os_3(CO)_9(\mu_3\text{-BCO})$ (I), a ketenylidene analogue, apparently through electrophilic attack at the oxygen of the unique carbonyl. Reactions of I with BX_3 (X = Cl, Br) result in an exchange of B and C atom positions in the BCO unit to form a vinylidene analogue, $(\mu\text{-H})_3Os_3(CO)_9(\mu_3\text{-CBX}_2)$. Boron-10-labeling experiments indicate that this transformation occurs through an intramolecular interchange of the boron and carbon atom positions. The ketenylidene analogue $(\mu\text{-H})_3Os_3(CO)_8(PPh_3)(\mu_3\text{-BCO})$ reacts similarly with BCl_3 to produce $(\mu\text{-H})_3Os_3(CO)_8(PPh_3)(\mu_3\text{-CBCl}_2)$. The nucleophiles PMe_3, PPh_3, and NMe_3 add to the tricoordinate boron of $(\mu\text{-H})_3Os_3(CO)_9(\mu_3\text{-CBCl}_2)$. Above −10 °C the NMe_3 adduct is converted to the salt $[NMe_3H][(\mu\text{-H})_2Os_3(CO)_9(\mu_3\text{-CBCl}_2)]$ and the PMe_3 adduct dissociates. The PPh_3 adduct decomposes above 30 °C. The reaction of $(\mu\text{-H})_3Os_3(CO)_9(\mu_3\text{-BCO})$ with $THF\cdot BH_3$ produces $(\mu\text{-H})_3Os_3(CO)_9(\mu_3\text{-BCH}_2)$, a second type of vinylidene analogue. In this case the boron and carbon atoms do not change positions. Alkyne analogues, $(\mu\text{-H})_3Os_3(CO)_9[\mu_3\text{-}\eta^2\text{-C(OBC}_8H_{14})B(Cl)]$ and $(\mu\text{-H})_3Os_3(CO)_9[\mu_3\text{-}\eta^2\text{-C[OB(Ph)Cl]B(Cl)}]$ are obtained from reactions of $(\mu\text{-H})_3Os_3(CO)_9(\mu_3\text{-BCO})$ with *B*-Cl-9-BBN and $PhBCl_2$. These compounds react with BCl_3 to produce $(\mu\text{-H})_3Os_3(CO)_9(\mu_3\text{-CBCl}_2)$. They also react with HCl to produce $(\mu\text{-H})_3Os_3(CO)_9(\mu_3\text{-CH})$.

Introduction

Metal ketenylidene clusters possess a rich and diverse chemistry. Cationic,[1,2] $[Co_3(CO)_9(\mu_3\text{-CCO})]^+$, $[CpMoCo_2(CO)_9(\mu_3\text{-CCO})]^+$, and neutral,[3,4] $(\mu\text{-H})_2M_3(CO)_9(\mu_3\text{-CCO})$ (M = Ru, Os), ketenylidenes react with nucleophilic reagents. The most extensively studied of these clusters, $[Co_3(CO)_9(\mu_3\text{-CCO})]^+$, has been shown by Seyferth and co-workers[1] to undergo nucleophilic attack exclusively at the β-carbon atom of the CCO unit. Monoanionic ketenylidenes, $[Fe_2Co(CO)_9(\mu_3\text{-CCO})]^-$ and $[(\mu\text{-H})Ru_3(CO)_9(\mu_3\text{-CCO})]^-$, exhibit similar reactivity.[5,6]

Shriver and co-workers[6-9] have demonstrated that the group VIII dianionic ketenylidenes $[M_3(CO)_9(\mu_3\text{-CCO})]^{2-}$ (M = Fe,

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