of Olah's reagent with organometallic compounds could afford a much-needed, efficient general route to transition metal fluoro complexes. There is a vast array of starting materials, but few reagents useful for making M-F bonds. Neither in a recent review[42] nor in our own examination of the literature could we find any precedent for the employment of Olah's reagent to convert metal-alkyl to metal-fluorine bonds. We proffer the suggestion that Olah's reagent may also be useful for the conversion of M-OR or M-H bonds to M-F bonds, whereby ROH or H_2, respectively, would be released.

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Registry No. 1, 138630-82-7; 2, 138630-83-8; $Mo_2Me_4(PMe_3)_4$, 64376-69-8; $Mo_2Me_4(PMe_2Ph)_4$, 67619-18-5; Mo, 7439-98-7.

Supplementary Material Available: A table of anisotropic thermal parameters (1 page); a listing of structure factors (1 page). Ordering information is given on any current masthead page.

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Spectroelectrochemistry of Copper-Zinc Superoxide Dismutase

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The temperature dependence of the reduction potential of bovine copper-zinc superoxide dismutase (CuZnSOD) has been determined at pH 7.0, with potassium ferricyanide as the mediator. The following thermodynamic parameters were obtained: $E^{\circ\prime} = 403 \pm 5$ mV vs NHE, $\Delta G^{\circ\prime} = -9.31$ kcal/mol (pH 7.0, 25 °C, μ 0.1 M); $\Delta H^{\circ\prime} = -21.4$ kcal/mol, $\Delta S^{\circ\prime} = -40.7$ eu, $\Delta S^{\circ\prime}_{rc} = -25.1$ eu. The large negative enthalpy of reduction may reflect the factors that are responsible for the unusually low reactivity of the enzyme with outer-sphere electron-transfer reagents.

Introduction

Bovine copper-zinc superoxide dismutase (CuZnSOD) is a dimeric enzyme of molecular weight 31 200 Da, containing one copper(II) and zinc(II) per monomer subunit.[1-4] According to the results of an X-ray crystal structure study of the oxidized (Cu^{II}) form of the enzyme at 2-Å resolution,[5-7] the copper site has a highly distorted square-pyramidal geometry with four histidine imidazoles and a water molecule as ligands, the latter occupying the apical position. One of the histidine imidazoles is deprotonated and acts as a bridging ligand between Cu^{II} and Zn^{II} in the nearby zinc site. The zinc site has a roughly tetrahedral geometry with the bridging imidazolate, two unshared histidine imidazoles, and an aspartate carboxylate making up the four ligands. The copper binding site in each monomer is located at the bottom of a narrow channel that is approximately 12 Å deep. Several charged residues on the surface of the protein and in the active-site channel appear to be important in directing superoxide and other anions toward the copper ion.[6,7] Catalysis of superoxide disproportionation occurs at the copper, which is accessible to solvent; the zinc ion seems primarily to play a structural role and is inaccessible to solvent.[1-4] The mechanism proposed for this enzyme involves reduction of Cu^{II} by one superoxide followed by oxidation of Cu^{I} back to Cu^{II} by a second superoxide. The Cu^{II} ions in the two subunits of the protein dimer are separated by 34 Å and appear to function independently.[1-4]

Reduction of the Cu^{II} form of CuZnSOD to give the Cu^{I} enzyme is accompanied by dissociation of the bridging imidazolate ligand from Cu^{II} and protonation of the nitrogen that had been bound to copper. That same ligand, now converted from imidazolate to imidazole, remains bound to zinc.[8] The reduction potential of CuZnSOD has been reported to be in the range 280-420 mV vs NHE.[9,10] In spite of this relatively high reduction potential, work in our laboratory has shown that CuZnSOD is reduced very slowly by reagents such as ascorbate and $Fe(CN)_6^{4-}$.[11] By contrast, the reaction of superoxide with either the oxidized or reduced form of CuZnSOD is known to occur with a rate near the diffusion limit.[12]

We undertook the study described here in order to characterize more fully the redox thermodynamics of CuZnSOD and to relate this new information to the observed kinetic properties. We used the technique of visible spectroelectrochemistry to measure the reduction potential and its temperature dependence, thereby determining the enthalpy and entropy of the reaction.[13] The results of this study are described here along with a discussion of how they may be related to the kinetics of the redox reactions of CuZnSOD.

Experimental Section

Materials. CuZnSOD isolated from bovine liver was purchased from Diagnostic Data, Inc. (Mountain View, CA). All other materials were prepared by standard procedures or used as supplied.[11]

Methods and Instrumentation. Long-path spectroelectrochemical measurements were performed by using 2-cm optical path length cells, prepared by minor modifications of a previously reported design.[14] The working electrode was a piece of gold foil that lines three walls of the sample compartment; the foil was connected by conducting epoxy to a gold wire, which acted as the external contact. The working electrode compartment was 7.2-mm wide by 16.0-mm high. Potentials were applied with a Princeton Applied Research polarographic analyzer (Model 174A) and measured with a microvolt digital multimeter (Keithley 177). The reference electrode was a miniature SCE (Sargent-Welch), and the counter electrode was a platinum wire. Sample solutions were stirred during electrochemical equilibration via a pneumatic stirrer contained

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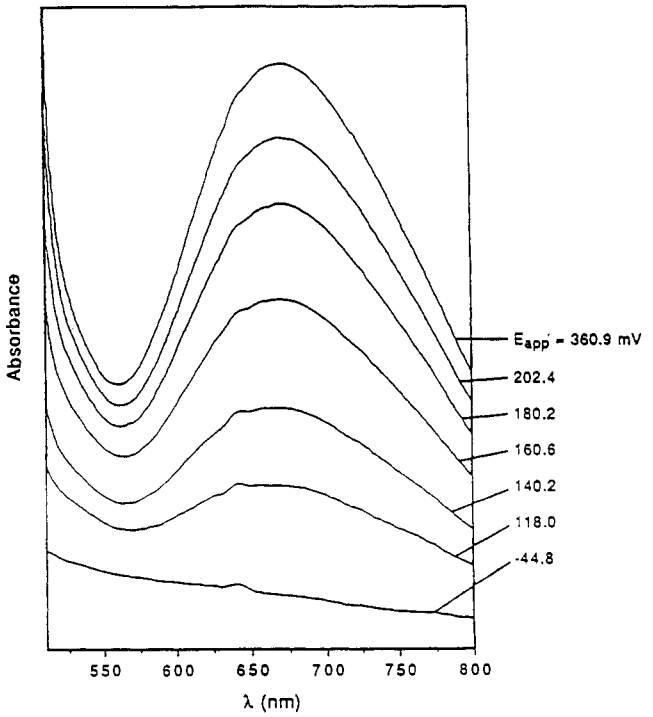

Figure 1. Overlay spectra obtained in the determination of the reduction potential of CuZnSOD (pH 7, 25 °C, μ 0.1 M, NaP_i). The applied potentials are relative to the SCE. The feature near 640 nm was apparent in the baseline spectrum.

in the shroud holder, which was mounted in the sample compartment of a Varian Cary 219 spectrophotometer. Sample temperature was monitored during each experiment by a Fluke digital thermometer (2175A) via a microthermocouple (Omega Engineering) placed inside the wall of the sample compartment. The digital thermometer readout was calibrated in a separate experiment by measuring the actual temperature of the solution in the cell using a second thermocouple probe.

The spectroelectrochemical runs were performed under anaerobic conditions, in order to avoid producing hydrogen peroxide during the experiments. H_2O_2 has been shown to inactivate CuZnSOD.[15] Samples were rigorously degassed and transferred to an inert-atmosphere (Ar) box (Vacuum Atmospheres), where they were loaded into the cell and mounted in a gastight stainless steel shroud. The shroud was then removed from the glovebox and placed in the spectrophotometer for the electrochemical experiment. The shroud and spectrophotometer mount have been described previously.[14]

Sample solutions were prepared to give a 3-fold excess of the mediator, $K_3Fe(CN)_6$, over SOD copper, with a total ΔA_{680} of 0.10–0.20 AU. Typical concentrations were [CuZnSOD] = 0.32 mM and $[K_3Fe(CN)_6]$ = 1.9 mM. The reduction potential at each temperature was determined by varying the potential applied across the cell and allowing the mediator and protein to come to redox equilibrium at each potential. The approach to equilibrium and the ratio of oxidized to reduced CuZnSOD at each applied potential were determined by monitoring the absorbance at 680 nm. Data points were fit to the Nernst equation to obtain the reduction potential. At least five points were obtained for each plot, in addition to the absorbance readings for the fully oxidized and fully reduced enzyme.

Enclosing the sample cell and reference electrode compartment in the shroud to maintain anaerobic conditions results in partial thermostating of the SCE. Thus, the temperature of the SCE at each cell temperature was determined in control experiments, and the observed CuZnSOD potentials were corrected for the variation in SCE potential with temperature before the thermodynamics calculations were performed.

Results

The results of a typical experiment are shown in Figure 1. The ratios of the concentrations of oxidized and reduced CuZnSOD determined from the absorbance at each applied potential are shown in Figure 2. A least-squares analysis of the data yields $E^{\circ\prime}$ = 403 mV vs NHE (pH 7.0, 25 °C, μ 0.10 M); the error is estimated to be ±5 mV. This potential is at the high end of the

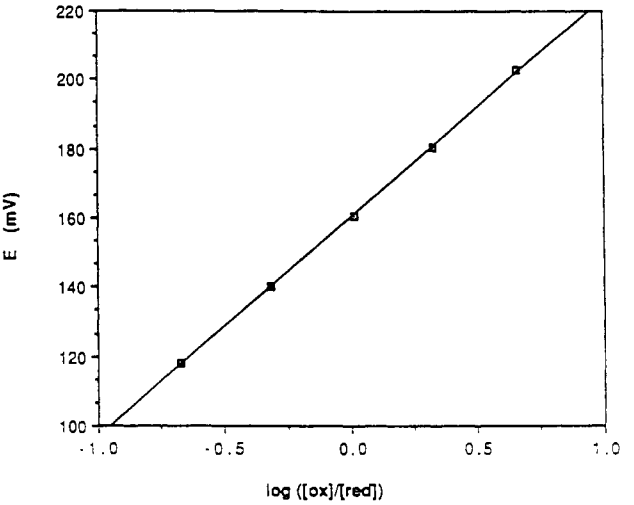

Figure 2. Nernst plot of the data in Figure 1. A value of $E^{\circ\prime}$ = 159 mV vs SCE was obtained from a least-squares fit of the data.

280–420-mV range of previously reported values for $E^{\circ\prime}$.[9,10]

It has been established by differential scanning calorimetry that ferricyanide decreases the melting temperature of CuZnSOD, thereby indicating that the stability of the enzyme is affected by this anion.[16] On the basis of coulometric titrations, Lawrence and Sawyer suggested that ferricyanide binds to one copper ion per protein dimer, causing the copper to be nontitratable.[10] (However, the protein used in the latter study was heterogeneous and exhibited anomalous behavior during coulometric titration with methylviologen as well as with ferricyanide.) In our experiments, no change in the visible spectrum of the protein occurred that could be attributed to perturbation of the copper site by ferricyanide, either on addition of ferricyanide to the solution or during the electrochemical experiments.

Other experiments also indicate that the presence of ferricyanide does not affect the reduction potential of CuZnSOD.[11] Oxidized CuZnSOD is reduced completely both by $Ru(NH_3)_6^{2+}$ (E° = 60 mV) and $Ru(NH_3)_5(py)^{2+}$ (E° = 260 mV),[17] with reduction by $Ru(NH_3)_6^{2+}$ faster than by $Ru(NH_3)_5(py)^{2+}$. Complete reduction by $Ru(NH_3)_5(py)^{2+}$ indicates that the CuZnSOD reduction potential is at least 350 mV, and the limited data obtained from spectroelectrochemical experiments with $[Ru(NH_3)_6]Cl_3$, $[Ru(NH_3)_5(py)]Cl_3$, and $[Co(phen)_3]Cl_3$ as mediators[18] are consistent with a CuZnSOD potential near 400 mV vs NHE.

As a control, the reduction potential of CuZnSOD was measured with cacodylic acid (dimethylarsenic acid) as buffer. An $E^{\circ\prime}$ of 400 ± 5 mV vs NHE (pH 7.0, 25 °C) was found,[11] which is within the error of the potential determined in phosphate buffer. Perturbation of the reduction potential by the buffer was of concern, because phosphate has been reported to bind to CuZnSOD.[19]

Reduction potentials of CuZnSOD in the range 0.7–37.2 °C are given in Figure 3. A fresh protein sample was used for each experiment, owing to the long equilibration time required at each applied potential. Thermodynamic parameters are as follows (pH 7.0, 25 °C, μ 0.1 M, NaP_i):[11] $\Delta G^{\circ\prime}$ = −9.31 kcal/mol; $\Delta H^{\circ\prime}$ = −21.4 kcal/mol, $\Delta S^{\circ\prime}$ = −40.7 eu, $\Delta S^{\circ\prime}_{rc} = S^{\circ\prime}_{red} - S^{\circ\prime}_{ox}$ = −25.1 eu.

Discussion

Substantial reorganization of the copper site is known to occur on reduction (e.g., Cu–Im^- bond cleavage, followed by protonation

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Table I. Thermodynamic Parameters at pH 7 and 25 °C

protein	$E^{\circ\prime}$ mV vs NHE	$\Delta S^{\circ\prime}$, eu	$\Delta S^{\circ\prime}_{rc}$, eu	$\Delta H^{\circ\prime}$, kcal/mol	ΔG°, kcal/mol
azurin[a] (*Pseudomonas aeruginosa*)	308	−31.7 ± 1.2	−16.1 ± 1.2	−16.6 ± 0.4	−7.10 ± 0.05
plastocyanin[a] (*Phaseolus vulgaris*)	360	−18.0 ± 1.2	−2.4 ± 1.2	−13.7 ± 0.4	−8.3 ± 0.05
myoglobin[b] (sperm whale)	60	−39.2 ± 1.2	−23.6 ± 1.2	−13.0 ± 0.4	−1.36 ± 0.05
cytochrome *c*[a] (horse heart)	270	−30.5 ± 1.2	−14.9 ± 1.2	−15.3 ± 0.4	−6.23 ± 0.05
CuZnSOD	403	−40.7 ± 4.4	−25.1 ± 4.4	−21.4 ± 1.4	−9.31 ± 0.1

[a] From ref 13. [b] From ref 14.

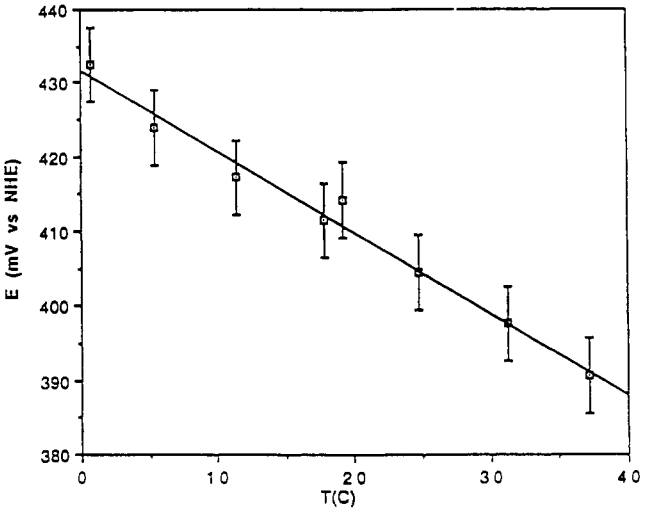

Figure 3. Temperature dependence of the reduction potential of CuZnSOD. E (mV vs NHE), °C: 432.5, 0.7; 423.9, 5.4; 417.2, 11.4; 411.5, 17.9; 404.5, 24.8; 395.0, 30.7; 397.6, 31.3; 390.6, 37.2 (pH 7.0, μ 0.1 M, NaP_i).

of Im^-). In addition, the Cu–OH_2 bond may break on reduction, although the evidence for this change is less conclusive.[20] The large negative entropy of reduction of CuZnSOD is difficult to understand in terms of these structural changes. According to EXAFS results,[20] the three remaining histidine ligands move in slightly (~0.05 Å) when the copper is reduced. Although it is likely that the binding of a proton on reduction will lower the entropy, breaking the Cu–His 61 and Cu–OH_2 bonds will contribute positive terms unless the proton taken up by His 61 comes not from the solution but from another amino acid side chain.

The enthalpy change on reduction of CuZnSOD is much more negative than those of typical single-site iron and copper proteins (Table I).[13,14,21] It is likely that this large negative enthalpy is due mainly to the stabilization of Cu^I by the three shortened Cu–Im bonds and the protonation of His 61 that accompanies reduction. Metal binding studies[22] have demonstrated that the presence of Zn^{II} in the zinc site increases the affinity of the copper site for Ag^I but not for Cu^{II}. If we assume that the binding of Ag^I and Cu^I is similar, this result suggests that Zn^{II} binding at the zinc site stabilizes a protein configuration at the copper site that is particularly favorable for Cu^I binding.[22]

The slow rates of reduction and oxidation of CuZnSOD by transition-metal complexes[11] can be partially attributed to the copper-site reorganization that accompanies electron transfer. Another reason for the slow rates may be that, while superoxide can diffuse into the narrow (~4-Å wide) active-site channel, most redox reagents cannot; thus, an electron must be transferred over a long molecular distance (>5 Å). Very slow rates are predicted for long-range electron transfer at relatively low driving forces to a redox center with a high reorganization energy.[23] Negatively charged reagents such as ferricyanide may bind to positively charged residues near the active-site channel, but the electron will still need to travel several angstroms through the channel.

Metalloproteins such as cytochrome *c*[24] and laccase[25] react very quickly with one superoxide ion but are then reoxidized by H_2O_2 or O_2 much faster than by O_2^-. In contrast, reduced CuZnSOD is rapidly reoxidized by O_2^-. According to the proposed mechanism, this specificity of the enzyme for superoxide is a crucial aspect of its role as a superoxide dismutase.[3] Thus, the inertness of CuZnSOD toward most redox reagents may reflect an important functional adaptation of the enzyme; this inertness protects it from intracellular redox reagents other than superoxide.

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