Redox Chemistry of Superoxide Dismutase. Cyclic Voltammetry of Wild-Type Enzymes and Mutants on Functionally Relevant Residues

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The reduction potential of human cuprozinc superoxide dismutase and of several of its functionally relevant mutants have been measured through cyclic voltammetry. The reduction potential of the bovine enzyme has been also measured and compared with literature values. The human enzyme has a slightly higher redox potential than the bovine isoenzyme ($E°$ = 0.36 ± 0.01 and 0.32 ± 0.01 V vs NHE, at pH 7.4, respectively). The redox properties of the bovine copper–cobalt derivative are very similar to those of the native protein. The pH dependence of the $E°$ value in the wild-type enzyme and its pH independence in the Asn-124 mutant, which has an empty zinc binding site over the entire pH range, is ascribed to the uptake of a proton by His-63 upon reduction. A pK_a value of 10.8 for this group is obtained from ^{1}H NMR titrations. It is proposed that also in the zinc-deprived derivative the copper–His-63 bond is broken upon reduction. Sizably negative reduction potentials were estimated for CN^- and N_3^--inhibited enzymes. The values are below the reduction potential of dioxygen to superoxide.

Introduction

Copper(II)–zinc(II) superoxide dismutases (SOD) are metalloenzymes formed by two identical subunits, each containing one copper(II) and one zinc(II) ion.[1-3] Copper and zinc are connected by a histidine imidazolate bridge. The chemical, structural, and catalytical properties of SOD have been extensively characterized through a variety of spectroscopic techniques[4-10] and X-ray studies[3,11] as well as through substitution of relevant amino acid residues by site-directed mutagenesis.[12-18]

The role of SOD seems to be that of catalyzing the dismutation of superoxide anion, strongly cytotoxic, to dioxygen and hydrogen peroxide.[1,4,19-22] The reaction has been proposed to occur through a two-step mechanism, in which the first step is the reduction of copper(II) to copper(I) with the formation of dioxygen. A second equivalent of superoxide then reoxidizes copper(I) to copper(II) with the release of hydrogen peroxide.[20,23,24] In an alternative mechanism,[25,26] the protein may stabilize O_2^- by bridging it between copper(II) and Arg-143, to favor its protonation and subsequent reduction to hydrogen peroxide by a second O_2^- ion which is oxidized to dioxygen.

In the first mechanism the value of the reduction potential is of fundamental importance. For instance, it has to be intermediate between the $E°$ of the semireactions $O_2 + e^- \rightarrow O_2^-$ (–0.16 V (referred to unit O_2 activity)) and $O_2^- + e^- + 2H^+ \rightarrow H_2O_2$ (+0.89 V). In the second case, copper(II) does not undergo reduction during the catalytic cycle. Nevertheless, the understanding of its redox properties is helpful in understanding, for instance, its pH-dependent properties. Recently, a reduction potential value of 0.403 ± 0.005 V vs NHE at pH 7 has been obtained for the bovine enzyme through visible spectroelectrochemistry, along with the corresponding thermodynamic parameters.[27] Potentiometric studies carried out in the pH 7–9 range, yielded potential values that showed a certain variability depending on both protein preparation and redox titrant. In particular, $E°$ values of +0.33 and +0.26 V were obtained at pH 7.4 by coulometric titrations with methyl viologen (MV^{2+}) for commercial and noncommercial enzymes, respectively, while $E°$ values of +0.40 and +0.25 V were determined at the same pH for the latter species through back-titration of the reduced protein with $K_3Fe(CN)_6$ and K_2IrCl_6, respectively.[28,29] No data are available for the human enzyme.

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The redox potential decreases with pH.[28,29] This is accounted for by assuming that the reaction is

$$E\text{–}Cu^{2+} + 1e^- + H^+ \rightarrow H^+\text{–}E\text{–}Cu^+ \quad (1)$$

The proton is believed to bind His-63, which is bridging the metals in the oxidized form and is detached from the copper ion in the reduced form.[4,11,29-33] The limited pH range investigated in the previous reports prevented the authors to determine any pK_a value.

Cyanide and azide are reported to be inhibitors of the enzyme and to bind to the copper ion.[2,4,10] The redox potential of the cyanide adduct has been estimated to be lower than -0.44 V through potentiometric titrations with MV^{2+}.[29]

We here report the reduction potentials measured through cyclic voltammetry and differential pulse polarography of two wild-type SOD isoenzymes, of several mutants with different activities, and of cyanide and azide derivatives. One goal is the characterization of the pH-dependent electrochemical properties of wild-type enzymes and of the Asn-124 mutant. The latter species is a zinc-deprived derivative which is stable in the pH 6–11 range[17] at variance with the chemically obtained Cu_2E_2 derivative which undergoes copper migration at the zinc site above pH 7[33] and whose redox potentials are also reported here. The lack of zinc in the Asn-124 mutant allows us to study this system, which is protonated at His-63, above pH 5–6 and to verify the mechanism for the pH-dependent properties of SOD.

A preliminary account of the application of cyclic voltammetry to SOD is reported in ref 34.

Experimental Section

Materials. Bovine liver erythrocyte SOD (BESOD) was purchased from Diagnostic Data Inc., Mountain View, CA, and used without further purification. The purity of the enzyme was checked by the A_{256}/A_{680} ratio. Human SOD (HSOD) and its AS mutant (HSOD-AS) were expressed in yeast and purified to homogeneity as previously reported.[12,35] HSOD-AS contains an alanine and a serine instead of two cysteine residues in position 6 and 111, respectively. These substitutions, though leaving almost unaltered the catalytic and structural properties of the enzyme, improve its thermal and chemical stability. All the mutants cited in this paper contain additional residue substitution(s), with which they are identified. The mutants on positions 124 and 137 were prepared with the procedure previously reported[17,18] as well as those on positions 132, 133,[36] and 136.[37] The Cu_2Co_2SOD derivative was prepared as described elsewhere.[19,38]

Electrochemical Measurements. Cyclic voltammetry (CV) and differential pulse polarography (DPP) were carried out on an Amel 472 Multipolarograph, while tensammetric curves were obtained on an Amel 468 AC Multipolarograph. Measurements were carried out with a gold working electrode (Amel), in the presence of 1,2-bis(4-pyridyl)ethene (Aldrich) as a promoter,[34] using a Pt sheet as counter electrode and a SCE as a reference. All the potentials reported in this paper are referenced to NHE. Potentials were calibrated against the MV^{2+}/MV^+ couple. A cell with a small volume sample ($V \sim 0.5$ mL) working under argon atmosphere at 25 ± 0.1 °C was used. Unbuffered protein solutions were freshly prepared before use and their concentration (in the 0.5–1 mM range) was determined spectrophotometrically ($\epsilon_{680} = 300\ M^{-1}\ cm^{-1}$). 0.1 M $NaClO_4$ was used as base electrolyte. CV and DPP measurements were performed at 200 and 2 mV s^{-1}, respectively, with a modulation amplitude of 25 mV and 0.5-s drop time. Conditions for tensammetric measurements were as follows: scan rate, 2 mV s^{-1}; frequency, 100 Hz; pulse height, 50 mV. In DPP experiments the $E_{1/2}$ values were obtained by applying the Parry–Osteryoung relationship: $E_p = E_{1/2} - \Delta E/2$ that relates the peak potential E_p to the half-wave potential $E_{1/2}$ for a given pulse amplitude ΔE. pH values were changed by adding small amounts of concentrated NaOH or $HClO_4$ under fast stirring. All the experiments were repeated several times and the values of the reduction potentials were found to be reproducible within ±10 mV.

Spectroscopic Measurements. Electronic spectra were collected on a Cecil 6600 spectrophotometer. EPR spectra were recorded at room temperature with a Bruker ER 200 D-SRC instrument working at 9.543 GHz. ^1H NMR spectra on the Cu_2Co_2 derivatives were collected, as previously described,[39] with a Bruker MSL 200. The 600-MHz ^1H NMR spectra were recorded on a Bruker AMX 600 spectrometer.

Considerations on the Electrochemical Methods

Cyclic voltammetry is being more and more exploited for the obtainment of electrochemical information on metalloproteins, although it is not yet of routine use. Its advantages and pitfalls have been discussed.[40] In most cases an electrochemical response can be obtained only with the use of a promoter.[40] 1,2-Bis(4-pyridyl)ethene proved to be the most effective for SOD.[34] It is strongly adsorbed onto the electrode surface and already yields a plateau value for the current at a 10^{-4} M concentration. Therefore, we are observing direct electrochemistry on a functionalized electrode. The experimental conditions for obtaining a well-defined signal depend critically on a number of parameters. In particular, CV measurements were carried out with a scan rate of 200 mV s^{-1} that was found to give the best compromise between the intensity of the currents and the reversibility of the process. The treatment of the working gold electrode is also a crucial factor: it must be accurately polished before each use with fine alumina (particle size about 0.075 μm, BDH) and successively treated in an ultrasonic pool (about 5 min). Nevertheless, cathodic and anodic waves show a distorted shape. This is not uncommon for proteins of this size. A contribution to the distortion from a possible potential-dependent reorientation of the promoter onto the electrode cannot also be excluded.[41,42] With such curves it is imperative to determine what is actually due to the metal center of the enzyme and what is not. Furthermore, the possible influence of the adsorption of the protein onto the electrode and of coupled chemical reactions must be carefully considered. The analysis of a series of experimental data, reported below, indicates that we are actually measuring electrochemistry on the bulk SOD. The cyclic voltammograms of HSOD (I), of apo-HSOD (II), and of a blank solution containing only the base electrolyte and the promoter (III) are shown in Figure 1. It is clear that the electrochemical process is due to the Cu(II) ion of the protein. The peak currents were

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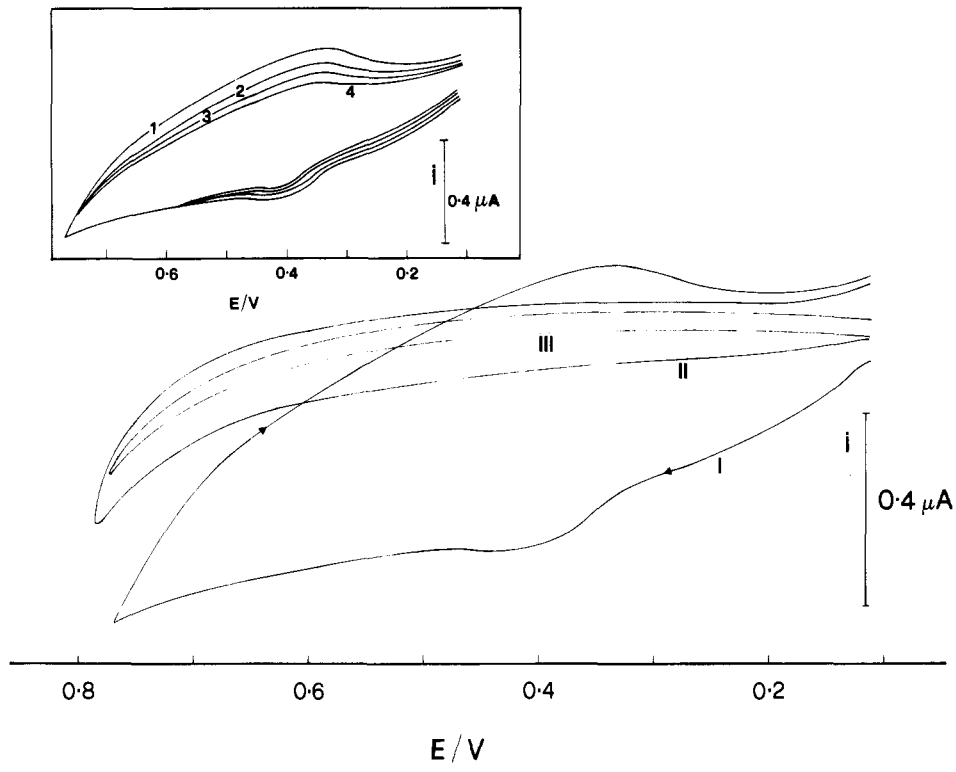

Figure 1. Cyclic voltammograms of (I) 10^{-3} M HSOD in the presence of 10^{-4} M 1,2-bis(4-pyridyl)ethene as a promoter and 0.1 M $NaClO_4$ as base electrolyte, (II) 10^{-3} M apo-SOD under the same conditions, and (III) a blank solution containing only the promoter and the base electrolyte. Conditions: scan rate, 200 mV s^{-1}; pH, 7.42; T = 25 °C. The inset shows voltammograms obtained with different scan rates: (1) 200 mV s^{-1}; (2) 100 mV s^{-1}; (3) 50 mV s^{-1}; (4) 20 mV s^{-1}.

found proportional to both protein concentration and $v^{1/2}$ (v = sweep rate) (Figure 1, inset). This indicates a diffusion-controlled electrochemical process. The comparable current of the anodic and cathodic peaks and their separation of about 70–90 mV (depending on pH and scan rate) indicate that the electrochemical process is quasi-reversible. As a consequence, the $E_{1/2}$ values can be evaluated from the equation $(E_p^a + E_p^c)/2$ (where E_p^a and E_p^c are the anodic and cathodic peak potentials, respectively) and can be assumed to correspond to the $E°$ values. The $E°$ values were found to be independent of both protein concentration and sweep rate (in the range 5×10^{-4} to 2×10^{-3} M and 10–200 mV s^{-1}, respectively), and this makes the occurrence of coupled chemical reactions unlikely. Moreover, the overall features of the single peak observed in DPP measurements (Figure 2a) are consistent with the presence of a unique electrochemical process, in agreement with the CV data. Finally, the similarity of the shapes of the tensammetric curves obtained in the presence and absence of protein (Figure 2b) confirms that the electrochemical process is diffusion-controlled and that the protein is not appreciably adsorbed on the electrode. The $E°$ values obtained for HSOD, BESOD, and mutants at pH 7.4 (see below) rule out the possibility of the electrode reaction being due to Cu(II) ion removed from the protein.

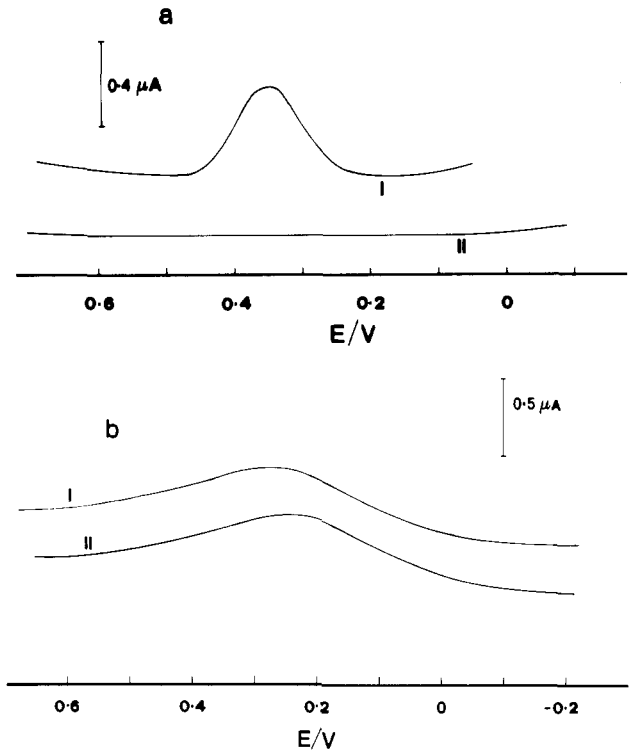

Figure 2. Differential pulse polarography (a) and tensammetric curves (b) of (I) 10^{-3} M HSOD in the presence of 10^{-4} M 1,2-bis(4-pyridyl)-ethene as a promoter and 0.1 M $NaClO_4$ as base electrolyte and (II) a blank solution containing only the promoter and the base electrolyte. DPP measurements were carried out with a scan rate of 2 mV s^{-1} at pH 7.42 and T = 25 °C. Conditions for the tensammetric measurements: scan rate, 2 mV s^{-1}; frequency, 100 Hz; pulse height, 50 mV; pH, 7.42; T = 25 °C.

Results

Cyclic voltammetry experiments were carried out on BESOD, HSOD, and HSOD-AS and on a number of mutants of the last species at a third amino acid position. A typical voltammogram is shown in Figure 1. At pH 7.4, $E°$ values of 0.32 ± 0.01, 0.36 ± 0.01, and 0.37 ± 0.01 V were determined for bovine, human, and human-AS enzyme, respectively.

The question of whether the promoter alters in some way the features of the active site must be addressed. In all cases, the addition of 1,2-bis(4-pyridyl)ethene leaves unchanged the electronic and EPR spectral features of the enzymes. Likewise, no changes have been detected in the isotropically shifted signals present in the ^1H NMR spectrum of the Cu_2Co_2SOD derivative. Substitution of the native zinc with cobalt(II) provides a derivative

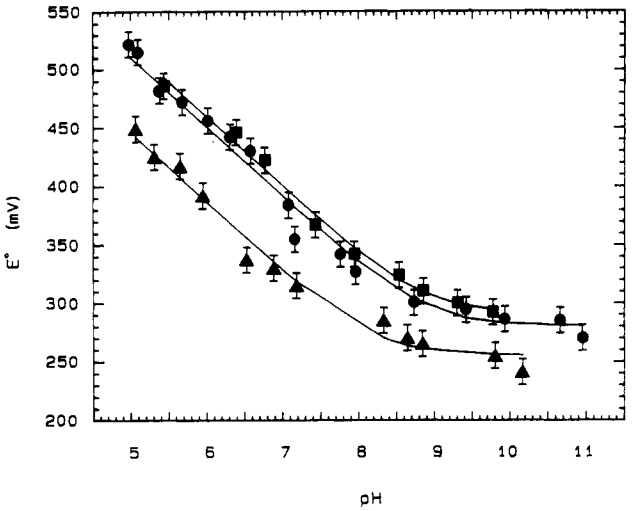

Figure 3. pH dependence of the redox potential for HSOD (●), HSOD-AS (■), and BESOD (▲).

which shows sharp and well-resolved ^{1}H NMR signals arising from protons of metal-binding residues and from others close to the metal centers. This ^{1}H NMR spectrum, fully assigned, is highly sensitive to any change in the active site.[43,44] Hence, a strong spectroscopic evidence is obtained that the promoter does not interact either with the metal(s) or with nearby residues but most probably interacts with a positive residue and/or with an hydrophobic region outside the active site, on the protein surface.

pH Dependence of the Redox Potentials. The pH-dependence of the $E°$ values for the three enzymes investigated is characterized by a linear decrease of the potential values on increasing pH from 5 to 9 (Figure 3). The slope of such a linear dependence (about 55 mV/pH) is consistent with the involvement of a one-proton acid–base equilibrium in the electrochemical process (eq 1). At pH values higher than 9 such a slope remarkably decreases along with the current values, especially those of the anodic peaks. The curves were thus fitted to the following single acid–base equilibrium equation:

$$E° = E°_{\lim} + 2.3\frac{RT}{F}\log\left(1 + \frac{[H^+]}{K_a}\right)$$

and pK_a values of 8.9 ± 0.6 (3σ), 8.8 ± 0.6, and 8.2 ± 0.6 were obtained for HSOD, HSOD-AS and BESOD, respectively. The best fit limit values at high pH were 0.28, 0.29, and 0.25 V, respectively. The pH-dependence of the potential values of the human enzyme in the presence of 0.05 M phosphate is very similar to that for the free enzyme.

It should be pointed out at this point that the pK_a values obtained from electrochemical measurements, although they are reproducible and have relatively low standard deviations, may differ from the thermodynamic value if the waves given by low- and high-pH species cannot be resolved (hence the measured potential is actually an average value),[45] as in the present case. It is difficult to estimate the magnitude of the effect, but for equilibria of the present kind, pK_a values lower than the thermodynamic values by 0.5–2 units can be observed.[45] Moreover, additional lowering effects on the observed pK_a values can arise from the adsorbed promoter and from additional adsorption of OH^- ions that increase the local pH value at the electrode–solution interface as compared to the bulk solution.[46] Overall, our pK_a values most probably represent lower limits. Since the measurements were performed under the same and rigorously controlled conditions, the difference between the pK_a values of the bovine and human isoenzymes is probably still meaningful. Potential values for the Cu_2Co_2–BESOD derivative were found to be slightly higher than those of BESOD (Table I).

Redox Potentials of Mutants. The reduction potentials of a series of mutants of HSOD-AS with specific aminoacid substitutions at the active site are reported in Table I. The locations of the various substituted residues with respect to the active site cavity are illustrated in Figure 4. The cyclic voltammetry experiments were carried out using the same conditions described above. Monoelectronic and quasi-reversible processes were invariably observed. The $E°$ values for all the mutants investigated are lower than that of the wild-type protein, independently of the nature of the substituted residue and of pH, and show linear pH-dependence parallel to that for the wild-type protein (Table I). Again, the relative changes from one mutant to another with respect to the wild-type protein are more reliable than the absolute values.

It is interesting to note that also the mutants in which a negative (Glu-132, Glu-133, or both) or a positive (Lys-136) residue is substituted with a neutral one (Figure 4) show a pH dependence of $E°$ similar to the wild-type protein. In particular, mutants at position 136 demonstrate that deprotonation of Lys-136 is not responsible for the observed pK_a in the redox potential (Figure 5). This finding is in agreement with other pH-dependent properties of the Lys-136 mutant[37] and with our interpretation of the electrochemical pK_a values (see below), while it does not support the proposed involvement of lysine residues in the regulation of the enzymatic activity.[47,48]

In contrast with all the other mutants, the $E°$ value for the Asn-124 mutant is basically independent of pH and close to the high-pH value for the wild type enzyme (Figure 6). The removal of the hydrogen-bonding bridge between the Cu ligand His-46 and the Zn ligand His-71, provided by Asp-124 (Figure 3) causes a decrease in the affinity of metals for the Zn site.[17] This makes the Asn-124 mutant a better model for the behavior of the wild-type protein deprived of the Zn^{2+} ion (Cu_2E_2SOD).[17] It is known, in fact, that in the Cu_2E_2SOD prepared chemically from the wild-type enzyme, the Cu^{2+} ion migrates to the zinc site at pH higher than 7.[9,33] Indeed, the latter species shows a behavior similar to that of Asn-124 mutant, but a further slight decrease in $E°$ is observed around pH 7–8 (Figure 6).

Anion Derivatives. Cyclic voltammograms performed on the adducts of bovine enzyme with azide and cyanide using the above conditions were of poor quality. However, differential pulse polarograms on a gold electrode, again in the presence of 1,2-bis(4-pyridyl)ethene as a promoter, gave fairly good signals. A cyanide concentration of 10^{-2} M and an N_3^- concentration of 10^{-1} M were found to give limiting potential values. Tensammetric measurements showed that in these conditions both anions do not alter appreciably the interfacial properties of the electrode in the presence of the promoter. Measurements with N_3^- concentrations ranging between 10^{-3} and 10^{-1} M show that the adduct formation is approximately halfway complete at 10^{-2} M N_3^-, consistent with the reported value for the affinity constant of the anion (94 ± 5 M^{-1}).[49] Potential values for the cyanide adduct at pH 10 and for the azide adduct at different pH values are reported in Table

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Table I. Electrochemical Potentials for Mutants of HSOD-AS[a] and for Derivatives of BESOD[b]

HSOD-AS mutant	pH	$E°$/V	HSOD-AS mutant	pH	$E°$/V
Ala-137 (Thr-137)	6.36	0.39 (0.45)	Ser-137 (Thr-137)	6.92	0.38 (0.41)
	7.05	0.35 (0.40)		7.48	0.34 (0.37)
	8.09	0.31 (0.34)		8.15	0.31 (0.34)
Ala-136 (Lys-136)	6.56	0.38 (0.43)	Gln-136 (Lys-136)	5.10	0.38 (0.51)
	7.04	0.35 (0.40)		6.24	0.33 (0.44)
	8.27	0.32 (0.33)		7.01	0.29 (0.40)
Gln-132 (Glu-132)	6.33	0.35 (0.45)	Gln-133 (Glu-133)	6.16	0.38 (0.45)
	7.11	0.32 (0.39)		7.37	0.33 (0.38)
	8.03	0.29 (0.34)		8.05	0.30 (0.34)
Gln-132, Gln-133	6.51	0.34 (0.43)			
(Glu-132, Glu-133)	7.45	0.30 (0.37)			
	8.33	0.26 (0.33)			
Cu_2E_2Asn-124	5.42	0.31 (0.49)	Cu_2E_2HSOD-AS	5.11	0.28 (0.51)
(Asp-124)	6.56	0.31 (0.43)		5.52	0.27 (0.49)
	7.21	0.30 (0.39)		6.19	0.28 (0.45)
	7.92	0.30 (0.35)		7.50	0.25 (0.37)
	8.88	0.31 (0.31)		8.35	0.24 (0.33)
	9.51	0.30 (0.30)		9.15	0.23 (0.30)
	10.02	0.29 (0.29)		10.01	0.22 (0.29)
	10.45	0.29 (0.29)		10.55	0.22 (0.29)
Cu_2Co_2BESOD	5.41	0.45 (0.42)			
	6.48	0.39 (0.36)			
	7.20	0.34 (0.32)			
	8.68	0.29 (0.26)			
BESOD + N_3^-	7.32	−0.22 (0.31)			
	8.25	−0.26 (0.28)			
	9.18	−0.31 (0.26)			
	10.27	−0.32 (0.25)			
BESOD + CN^-	10.18	−0.59 (0.25)			

[a] The substituted amino acid in HSOD-AS is indicated in parentheses along with the corresponding $E°$ values for HSOD-AS interpolated at the selected pH from Figure 2. [b] Values in brackets are referred to BESOD at the same pH values interpolated from Figure 2.

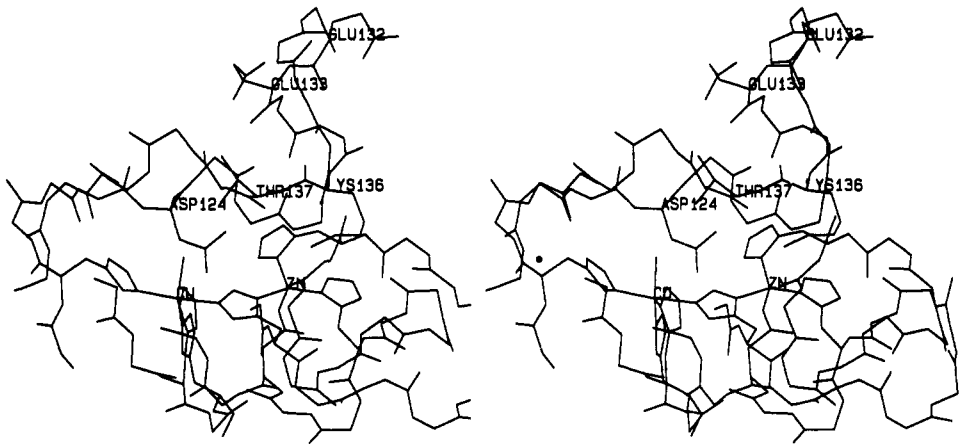

Figure 4. Stereoview of the active site of SOD taken from X-ray data on the bovine enzyme.[3] The labeled residues were those for which mutants are studied in the present work.

I. For the latter derivative measurements at pH lower than 7 were prevented by hydrogen discharge. It is apparent that the inhibited species show potential values dramatically different from those of the free enzyme. The value obtained for the cyanide adduct is consistent with that estimated from potentiometric titrations with MV^{2+} (<−0.44 V),[29] and the data for both derivatives are in line with the remarkable lowering of the reduction potential for the Cu(II)/Cu(I) couple in these species proposed from studies of electron transfer between BESOD and the ferro/ferricyanide couple.[50] For both derivatives the half-peak widths indicate a quasi reversible redox equilibrium.

Microscopic pK_a of His-63. A likely candidate for the group responsible of the pK_a around 9 of the $E°$ values is the His-63 residue (Figure 4) which is detached from the copper ion upon reduction.[4,11,29-33] We have therefore performed a ^{1}H NMR titration of the reduced wild type derivative to evaluate the microscopic pK_a value of His-63. The results are shown in Figure 7. The C2 proton of His-63[51] does undergo a pH transition typical of deprotonation of the ring nitrogen. The data are fitted to a pK_a of 10.8 ± 0.1 (3σ). This value is sizably higher than that obtained above from the $E°$ data, but still within the range of expected lowering of the latter pK_a from the thermodynamic value discussed above.[45,46]

Discussion

The absence of a direct electrochemical response for all the species of SOD investigated is the result of an electron transfer between the protein and the electrode surface intrinsically slow and/or hindered by the formation of an adsorbed layer of (denatured) protein on the electrode surface itself. Nitrogen-containing promoters are found effective in favoring such an electron exchange.[34] Wild-type human and human-AS protein show nearly overlapping $E°$ values over all the pH range

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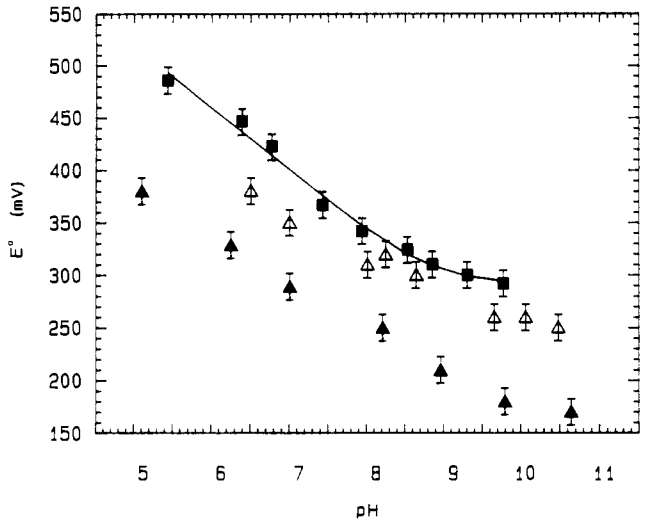


Figure 5. pH dependence of the redox potential for HSOD-AS (■), Ala-136 mutant (△), and Gln-136 mutant (▲).

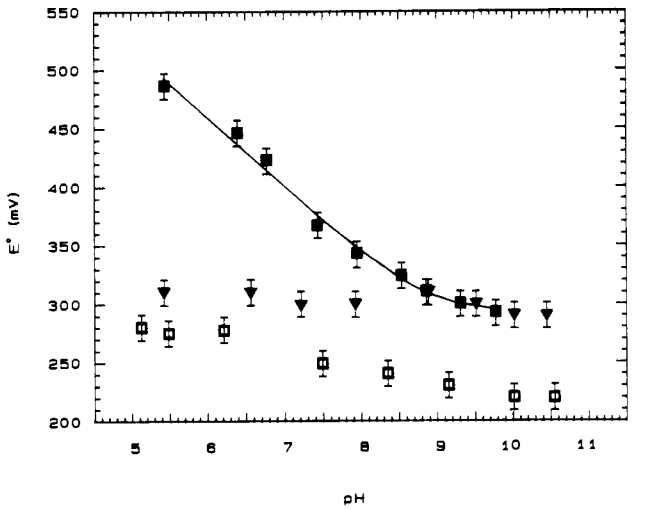


Figure 6. pH dependence of the redox potential for HSOD-AS (■), Cu_2E_2Asn-124 (▼), and Cu_2E_2 HSOD-AS (□).

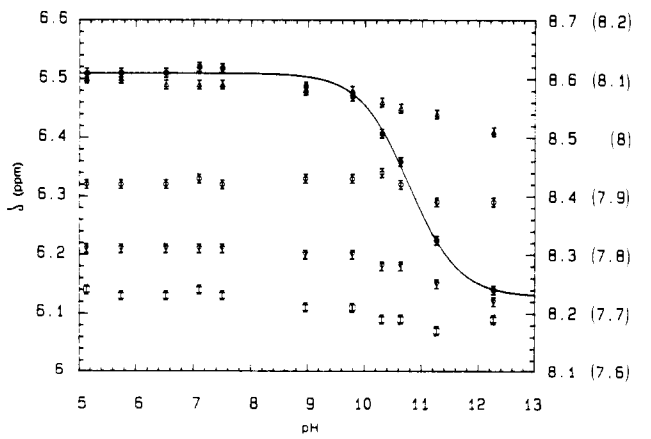
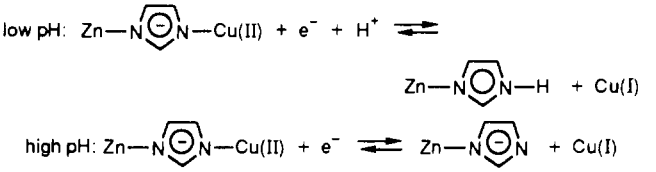


Figure 7. Plot of the ^1H NMR chemical shift (600 MHz, 27 °C) versus pH of the C-2 proton of the five metal binding histidines in $Cu^{I}Zn^{II}$SOD: (●) His-63; (△) His-80; (○) His-48; (▽) His-71; (□) His-120. The scale refers to His-63, while right scales are relative to His-80, to His-48 and His-120, and to His-71 (values in parentheses).

investigated, in line with the close similarity of their chemical behaviors, while the bovine enzyme invariably shows lower potential values. The value obtained for BESOD at pH 7 (0.330 ± 0.010 V) is lower than that recently determined through visible spectroelectrochemistry (0.403 ± 0.005 V).[27] Discrepancies of this order of magnitude between voltammetric and potentiometric values of the reduction potential of metalloproteins are largely documented[40] and arise primarily from the intrinsically different mechanism of the electron exchange which occurs in heterogeneous and homogeneous phases, respectively. Whichever comparison would be purely speculative. Different $E°$ values were also previously obtained from potentiometric titrations with MV^{2+} (+0.33 and +0.26 V at pH 7.4) depending on the protein preparation.[29] Again, values of +0.40 and +0.25 V were obtained for BESOD at pH 7.4 from titrations with $K_3Fe(CN)_6$ and K_2IrCl_6, respectively.[28,29] It is apparent that the potential value is affected by the nature of the mediator, which may possibly influence the stability of the protein. Moreover, evidence was obtained that the above anions act not only as simple redox partners but also interact closely with the copper centers, though the perturbation of the copper site by ferricyanide has been recently excluded.[27] Our data appear to be obtained on a protein neither perturbed by the promoter nor affected by adsorption phenomena onto the electrode. We have no elements for claiming our potential value more reliable than the others as the absolute value for the reduction potential of SOD. However, we stress again that, within the experimental error, the variations within our sets of data with pH, inhibitor binding to the protein, and mutations in the active site are real and show an inherent consistency with known properties of the enzyme.

From the present data no evidence is obtained for a previously proposed inequivalence of the two protein subunits:[29] more precisely, the shape of the voltammetric signal sets the upper value for the difference in the reduction potentials, if any, at about 0.03 V.

While the reduction potential of all the species investigated is found pH-dependent due to the involvement of one proton in the redox equilibrium, with a low-pH $E°$ value of about 0.45–0.50 V and a high-pH limit value of 0.24–0.25 V, respectively, the enzymatic activity of SOD is known to be pH-independent up to about pH 10, while with a further increase in pH it decreases with an approximately unit slope in log units.[15,16] Hence, the kinetics of SOD-catalyzed superoxide disproportionation is not affected at all by the relative thermodynamic stability of the redox states of the copper ion around neutral pH. Instead, it depends on the protonation state of a group whose pK_a is close to that regulating the redox potential. The pK_a values of 8.9, 8.8, and 8.2 determined here for HSOD, HSOD-AS, and BESOD, respectively, must be due to a group present in the reduced form and absent in the oxidized form. Most probably, this pK_a is relative to the deprotonation of the Zn-bound imidazole group of His-63 of the reduced form,[4,11,29-33] according to the following equilibria:



The case of the Asn-124 mutant is thus particularly interesting as far as the observed pK_a is concerned. In this species the zinc site is empty and, unlike the Cu_2E_2 derivative of the wild-type protein, the Cu^{2+} ion does not migrate in this site at alkaline pH. The $E°$ values are pH independent and leveled at the wild-type high-pH value. Apparently, the absence of zinc abolishes the group undergoing deprotonation in the wild-type reduced protein. This is a strong evidence in favor of His-63 as the candidate group. In Cu_2E_2 SOD His-63 is either coordinated to copper as the other three histidines or noncoordinated, given the preference of Cu(I) for low coordination numbers. In the first hypothesis

His-63 would be a normal ligand and therefore expected to have a lower pK_a when coordinated to Cu(II) than to Cu(I). Therefore, we would predict a pH-independent $E°$ value up to the pK_a of the oxidized form, after which the $E°$ values should decrease with pH. This is obviously not the case. In the second hypothesis His-63 would be a free residue, with a pK_a around 5–6 for the cationic to neutral form and then a pK_a around 14 for the neutral to anionic form. Therefore a pH independence of $E°$ is expected over the whole range from 5–6 to 14. This behavior is consistent with the experimental data. Accordingly, we propose that the structure of Cu(I) in Cu_2E_2SOD is the same as that in Cu_2-Zn_2SOD.

The residual pH dependence of $E°$ observed for the wild-type Cu_2E_2-derivative above pH 7 is most probably linked to the migration of the Cu^{2+} ion to the Zn^{2+} site that gives rise to exchanging species with a different site occupancy, each characterized by an individual reduction potential. Overall, the data on the Cu_2E_2 derivatives strongly support the assignment of the pK_a around 8–9 observed in the reduction potential of the holoenzyme to the ionization of the zinc-coordinated His-63 in the reduced protein.

NMR titration experiments, coupled with the full assignment of the active site protons recently performed,[51] have allowed us to determine the actual value of the microconstant for the deprotonation of His-63 in the reduced form. The determined value of 10.8 is sizably higher than that measured electrochemically, but still within the possible range of deviations due to the electrode-dependent phenomena mentioned above.

It is worth noting that the pK_a of 10.8 measured here for the deprotonation of His-63 in the reduced form is in the same ballpark as the pK_a measured for the loss of activity at high pH. Further experiments are, however, needed to establish whether this coincidence is just fortuitous or if indeed the enzyme requires a protonated His-63 to be active.

The redox potential for all the mutants investigated, bearing substitutions on most of the relevant residues in the active cavity resulted to be, even if to a different extent, invariably lower than those of the wild-type protein over all the pH range investigated. Such a decrease of $E°$ values for mutants having a Gln residue instead of a Glu residue in position 132 and/or position 133, and an Ala or Gln residue instead of Lys in position 136, can hardly be interpreted on simple electrostatic bases, since the above replacements cause opposite variation of charge in the proximity of the copper ion. Mutants on Thr-137 (Figure 4) show $E°$ values very close to that of the wild-type enzyme: substitutions with Ala and Ser scarcely affect the potential (see Table I), especially in the latter case, where the functionally relevant OH group is maintained. It should be noted that substitution of the positively charged Lys-136 residue with neutral residues maintain the pH dependence of the reduction potential. Since Lys-136 is the closest acid–base group to copper (besides His-63) (Figure 4), this is a further support for His-63 being the group responsible for the observed pH dependence.

The pattern of the redox potential for the mutants does not correlate with their activities. This finding is not surprising since the $E°$ values for the mutants remain well within the limits defined by the $E°$ values of the two catalyzed semireactions. The overall enzymatic catalysis is the result of many factors that can affect to a different extent the dismutation rate.

The metal derivative of BESOD with Co^{2+} inserted in the Zn^{2+} site has been deeply investigated through ^1H NMR spectroscopy and proved to be extremely informative on the structural features of the metal sites and on the binding mode of a number of inhibitors. That this information can be confidently transferred to the native enzyme is further demonstrated by the small changes of the redox potential of SOD induced by such a metal substitution. The slightly higher $E°$ values for the cobalt derivative, if significant, can be tentatively attributed to a shift of the His-63 pK_a toward higher values as the result of a lower binding strength toward the cobalt ion as compared to the zinc ion.

The dramatic changes of $E°$ upon binding of azide and cyanide clearly indicate that in both cases the anion binds to the copper ion, stabilizing the oxidized state. The extent of the potential lowering is such that in both cases the bound SOD would be unable to disproportionate the superoxide anion also from a purely thermodynamic point of view as its redox potential is no longer intermediate with those of the two pairs involved in the O_2^- dismutation.

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