Copper−Zinc Superoxide Dismutase: Why Not pH-Dependent?

Lisa M. Ellerby,[†] Diane E. Cabelli,*[,‡] Janet A. Graden,[†] and Joan Selverstone Valentine*[,†]

Contribution from the Department of Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, California 90095-1569, and Chemistry Department, Brookhaven National Laboratory, Upton, New York 11973

Received November 16, 1995[⊗]

Abstract: Copper−zinc superoxide dismutase (CuZnSOD) catalyzes the disproportionation of superoxide to hydrogen peroxide and dioxygen at diffusion controlled rates. Previous mechanistic studies have focused on the dramatic electrostatic guidance mechanism by which superoxide is drawn into the active site of this enzyme. Another striking but less understood feature of this enzyme is its ability to dismutate superoxide over a wide range of pH (5−9.5) without any change in rate-determining step or structural changes at the active site copper. To investigate the explanation for this pH independence, we have redetermined the rate of superoxide disproportionation, k_{cat}, catalyzed by the zinc-deficient form (Cu-apoSOD) of the enzyme as a function of pH and have found that it is pH-dependent, in contrast to the native enzyme, even under conditions in which the copper ion does not leave the native copper-binding site. In addition, we have determined the rate of reduction, k_1, of Cu-apoSOD by superoxide and have found that this step of the catalytic cycle is pH-independent. We conclude that the reoxidation rate, k_2, of the catalytic cycle is pH-dependent for Cu-apoSOD. These results have led us to propose that the key role of the zinc and of the histidyl imidazolate that bridges copper and zinc in CuZnSOD is to aid in the rapid dissociation of the product peroxide.

Introduction

Copper−zinc superoxide dismutase (CuZnSOD)[1] is a dimeric protein (MW = 31 200) with two identical subunits, each containing one Cu^{2+} and one Zn^{2+} ion, which catalyzes the dismutation of superoxide, O_2^-, to dioxygen and hydrogen peroxide with high efficiency.[2] Detailed mechanistic studies using pulse radiolysis have demonstrated that the copper center is cyclically reduced and oxidized by superoxide: superoxide first reduces the Cu^{II} center of CuZnSOD to produce dioxygen (reaction 1), and then another molecule of superoxide oxidizes the Cu^{I} center of CuZnSOD to produce hydrogen peroxide (reaction 2).[3−5]

$$O_2^- + Cu^{II}Zn^{II}SOD \xrightarrow{k_1} O_2 + Cu^{I}Zn^{II}SOD \quad (1)$$

$$O_2^- + 2H^+ + Cu^{I}Zn^{II}SOD \xrightarrow{k_2} H_2O_2 + Cu^{II}Zn^{II}SOD \quad (2)$$

The rate constants of the individual steps of this mechanism have been measured and found to be identical, i.e., $k_1 = k_2 = 2 \times 10^9\ M^{-1}\ s^{-1}$, and pH-independent over the pH range 5−9.5.[3,4] The high, nearly diffusion controlled, rates of reaction of both the oxidized and reduced forms of the enzyme with superoxide indicate that collisions of superoxide with the enzyme cannot be random since the access of the superoxide anion to the active site Cu^{II} is via a narrow channel. Theoretical, structural, and mechanistic studies have shown that superoxide anion is electrostatically guided into the active site by a positively charged channel.[6−9] In fact, recent studies have shown that the nearly diffusion controlled rate of superoxide dismutase can be improved upon by increasing the charge density of the electrostatic patch steering superoxide anion into the enzyme active site.[9,10]

Two other striking features of copper−zinc superoxide dismutase are the nearly pH independent rate of the CuZnSOD-catalyzed disproportionation of superoxide, k_{cat}, and the invariance of its spectroscopic properties from pH 5 to pH 9.5.[11,12] The latter property indicates that the identity of the copper site ligands and their geometry of binding to the Cu^{II} center in CuZnSOD remain unaltered. A key residue believed to function in maintaining the pH independence of the catalytic and spectroscopic properties of CuZnSOD is the bridging imidazolate which coordinates to both the Zn^{2+} and Cu^{2+}. Reduction of the Cu^{II} form of copper−zinc superoxide dismutase has been shown to occur with uptake of one proton per subunit.[13,14] This proton is believed to protonate the bridging imidazolate in association with the breaking of the bridge upon reduction of the copper (reaction 3). It has been postulated that the histidyl imidazole coordinated to zinc acts in the second step of the enzymatic redox cycle (reaction 4) to protonate the copper

[†] University of California, Los Angeles.
[‡] Brookhaven National Laboratory.
[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1996.

(1) Abbreviations: CuZnSOD represents the native form of copper−zinc superoxide dismutase. Cu-apoSOD represents zinc-deficient copper−zinc superoxide dismutase.

(2) Fridovich, I. *Annu. Rev. Biochem.* **1995**, *64*, 97−112.
(3) Rotilio, G.; Bray, R. C.; Fielden, E. M. *Biochim. Biophys. Acta* **1972**, *268*, 605−609.
(4) Klug-Roth, D.; Fridovich, I.; Rabani, J. *J. Am. Chem. Soc.* **1973**, *95*, 2786−2790.
(5) Fielden, E. M.; Roberts, P. B.; Bray, R. C.; Lowe, D. J.; Mautner, G. N.; Rotilio, G.; Calabrese, L. *Biochem. J.* **1974**, *139*, 49−60.
(6) Getzoff, E. D.; Tainer, J. A.; Weiner, P. K.; Kollman, P. A.; Richardson, J. S.; Richardson, D. C. *Nature* **1983**, *306*, 287−290.
(7) Cudd, A.; Fridovich, I. *J. Biol. Chem.* **1982**, *257*, 11443−11447.
(8) Cudd, A.; Fridovich, I. *FEBS Lett.* **1982**, *144*, 181−182.
(9) Getzoff, E. D.; Cabelli, D. E.; Fisher, C. L.; Parge, H. E.; Viezzoli, M. S.; Banci, L.; Hallewell, R. A. *Nature* **1992**, *358*, 347−351.
(10) Polticelli, F.; Bottaro, G.; Battistoni, A.; Carri, M. T.; Djinovic-Carugo, K.; Bolognesi, M.; O'Neill, P.; Rotilio, G.; Desideri, A. *Biochemistry* **1995**, *34*, 6043−6049.
(11) Rotilio, G.; Morpurgo, L.; Giovagnoli, C.; Calabrese, L.; Mondovi, B. *Biochemistry* **1971**, *10*, 2187−2192.
(12) Bannister, J. V.; Bannister, W. H.; Rotilio, G. *CRC Crit. Rev. Biochem.* **1987**, *22*, 111−180.
(13) Fee, J. A.; DiCorleto, P. E. *Biochemistry* **1973**, *12*, 4893−4899.
(14) Bailey, D. B.; Ellis, P. D.; Fee, J. A. *Biochemistry* **1980**, *19*, 591−596.

S0002-7863(95)03845-5 CCC: $12.00

$$\overline{Cu^{II}-Im-Zn^{II}SOD} + O_2^- + H^+ \xrightarrow{k_1} \overline{Cu^{I}\ Hlm-Zn^{II}SOD} + O_2 \quad (3)$$

$$\overline{Cu^{I}\ Hlm-Zn^{II}SOD} + O_2^- \xrightarrow{k_2} \overline{Cu^{II}-O_2^{2-}\ Hlm-Zn^{II}SOD} \xrightarrow{H^+} \overline{Cu^{II}-Im-Zn^{II}SOD} + H_2O_2 \quad (4)$$

peroxide species formed.[15] In contrast, under saturating conditions of superoxide, the turnover of the enzyme has been shown to be too fast for protonation and deprotonation of the bridging histidine to occur during the catalytic redox cycle.[16] The metal peroxide dianion $Cu^{II}-O_2^{2-}$ must be protonated via either solvent or the histidyl imidazole prior to its release from the copper center because peroxide dianion is highly basic and thus too unstable to be released free in its unprotonated form (reaction 4). The pH independence of the rates of these reactions indicates that diffusion remains rate limiting even at high pH where the concentration of protons is low.

We have recently reported on the properties of a mutant yeast CuZnSOD, H63ACuZnSOD, in which the bridging histidine (His63) had been converted to alanine using site-directed mutagenesis.[17] The enzymatic activity of this mutant enzyme was found to be markedly pH-dependent, with an activity that was 250-fold less than that of the wild type at physiological pH. We found the vis−UV and EPR spectral properties to be pH-dependent as well. In seeking the source of the pH dependence of the properties of this mutant enzyme, it became apparent to us that the pH dependence of the activity of bovine Cu-apoSOD, the zinc-deficient form of the enzyme, was very similar to that which we observed for H63ACuZnSOD in the low pH range (pH 5.0−8.0). In other words, removal of the imidazolate bridge, either by replacement of the bridging His63 with alanine or simply by removal of the Zn^{2+} ion, resulted in pH-dependent behavior. We therefore decided to reopen our earlier investigation of the pH-dependent behavior of bovine Cu-apoSOD.[18,19] Our results, which are presented below, have led us to a new mechanistic proposal concerning the role of the bridging imidazolate−zinc moiety of CuZnSOD.

Experimental Section

Sample Preparation. Bovine liver CuZnSOD was obtained from Diagnostic Data, Inc. (Mountain View, CA). The protein concentration of the native protein and the apoprotein were determined spectrophotometrically, with $\epsilon_{258\ nm} = 10\ 300$ and $2920\ M^{-1}\ cm^{-1}$, respectively.[20] The protein concentration was also determined using the Biorad method. The metal content of the protein derivatives was determined by atomic absorption (AA) analysis on a Varian spectAA-30 or Pye-Unicam or GBC instrument (error <5% for Cu or Zn). The preparation of apoSOD and Cu-apoSOD has been described previously.[20]

Kinetic Measurements. Pulse radiolysis experiments were carried out using the 2 MeV Van de Graaff accelerator at Brookhaven National Laboratory, New York, as described previously.[21] Dosimetry was carried out with KSCN, assuming $G((SCN)_2^-) = 6.13$ and $\epsilon_{472\ nm} = 7950\ M^{-1}\ cm^{-1}$. Superoxide radicals were generated in O_2-saturated aqueous solutions containing formate, and the concentration of O_2^- was determined from its absorbance at 245 nm ($\epsilon = 2350\ M^{-1}\ cm^{-1}$). Solutions contained 0.01 M formate (Sigma Chemical Co.) and 0.01−0.05 M potassium phosphate (Ultrex, J. T. Baker Chemical Co.). Some experiments also had 0.01 M sodium acetate and/or ethylenediaminetetraacetate (Sigma Chemical Co.), a metal ion chelator, present. The pH was adjusted by the addition of NaOH (GFS Chemical Co.; 99.999%) or H_2SO_4 (GFS Chemical Co.).

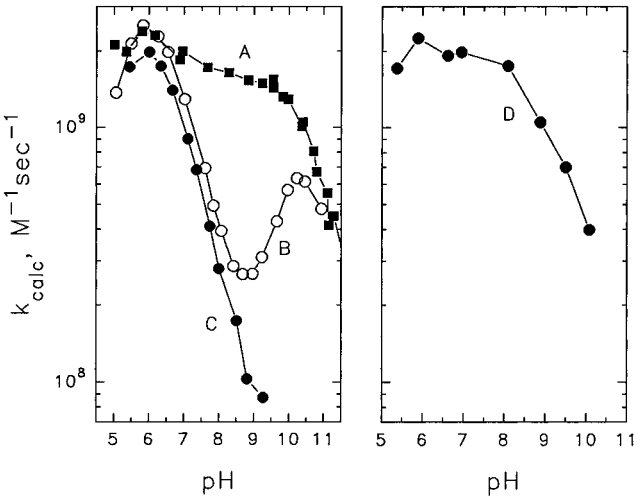


Figure 1. pH dependence of the specific activity of bovine CuZnSOD compared to that of Cu-apoSOD as measured by pulse radiolysis. (A)−(C) show the second-order rate constant, k_{cat}, as a function of pH, for catalytic dismutation of superoxide by the enzyme during turnover. (D) shows the rate constant for reduction of Cu(II)apoSOD, k_1, determined under conditions of excess enzyme relative to superoxide. The copper center of Cu-apoSOD was monitored spectroscopically at 680 nm for this latter experiment. Specific activity: (A) 0.3−3 μM CuZnSOD in 10 mM formate, 10 mM phosphate, (B) 1.0 μM Cu-apoSOD in 10 mM formate, 50 mM phosphate, (C) 1.0 μM Cu-apoSOD in 50 μM EDTA, 10 mM formate, 10 mM phosphate, and (D) 200 μM Cu-apoSOD in 10 mM formate, 10 mM sodium acetate.

The concentrations of enzyme and substrate were adjusted so as to allow us to determine both the rate of reduction (k_1) of the enzyme and the catalytic rate of dismutation (k_{cat}) of superoxide by the enzyme. Measurement of k_1 required that the concentration of the enzyme be substantially higher than that of superoxide in order to prevent enzyme turnover. This rate was measured (1) using concentrated enzyme (200 μM) and monitoring the disappearance of the Cu(II) absorption band at 680 nm ($\epsilon = 150\ M^{-1}\ cm^{-1}$) and (2) using lower concentrations of enzyme (10 and 25 μM), where the disappearance of superoxide could be monitored at 280−300 nm ($\epsilon = 400-800\ M^{-1}\ cm^{-1}$). In the latter experiment, the initial pulses of superoxide were relatively small (1−3 μM) and its disappearance was by reaction with Cu(II) only.

Measurements of k_{cat} were carried out under conditions where turnover was complete, either (1) where the enzyme concentration (1 μM) was much lower than that of superoxide or (2) at higher concentration (10 and 25 μM) where sufficient superoxide was delivered to the sample to drive the enzyme into complete turnover.

We attempted without success to measure the rate of reoxidation of the enzyme by superoxide. Since reduction by superoxide of the Cu(II) enzyme is much faster than reoxidation by superoxide of the Cu(I) enzyme, the determination of the latter can only be achieved by reducing the enzyme completely and then generating superoxide in the presence of a large excess of the enzyme. The reduction was carried out using CO_2^-, which is known to reduce the Cu(II) efficiently. However, the reoxidation rate must be measured at 680 nm, where the molar absorbance of the enzyme is very small. We found experimentally that we consequently could not determine k_2 without interference by k_1. Therefore, in the absence of a direct determination of k_2, we have inferred the reoxidation rate from k_{cat} and k_1. Knowing k_{cat} and k_1 and assuming the mechanism given by reactions 1 and 2, k_2 can be

(15) Hodgson, E. K.; Fridovich, I. *Biochemistry* **1975**, *14*, 5294−5303.
(16) Fee, J. A.; Bull, C. *J. Biol. Chem.* **1986**, *261*, 13000−13005.
(17) Graden, J. A.; Ellerby, L. M.; Roe, J. A.; Valentine, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 9743−9744.
(18) Pantoliano, M. W.; Valentine, J. S.; Burger, A. R.; Lippard, S. J. *J. Inorg. Biochem.* **1982**, *17*, 325−341.
(19) Valentine, J. S.; Pantoliano, M. W.; McDonnell, P. J.; Burger, A. R.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4245−4249.
(20) Pantoliano, M. W.; Valentine, J. S.; Nafie, L. A. *J. Am. Chem. Soc.* **1982**, *104*, 6310−6317.
(21) Cabelli, D. E.; Bielski, B. H. J. *J. Phys. Chem.* **1983**, *87*, 1809−1812.

calculated from $k_2 = 1/2k_{cat}$. The kinetic derivation for these various conditions is summarized below and follows from reactions 5 and 6:

$$O_2^- + Cu^{2+}SOD \xrightarrow{k_1} O_2 + Cu^{+}SOD \quad (5)$$

$$O_2^- + Cu^{+}SOD \xrightarrow{k_2,\ 2H^+} H_2O_2 + Cu^{2+}SOD \quad (6)$$

$$-d[O_2^-]/dt = k_1[O_2^-][Cu^{2+}SOD] + k_2[O_2^-][Cu^{+}SOD] = [O_2^-]\{k_1[Cu^{2+}SOD] + k_2[Cu^{+}SOD]\} \quad (7)$$

$$-d[O_2^-]/[O_2^-]dt = k_{obs}\ (s^{-1}) = \{k_1[Cu^{2+}SOD] + k_2[Cu^{+}SOD]\} \quad (8)$$

where, under catalytic (turnover) conditions

$$k_1[Cu^{2+}SOD] = k_2[Cu^{+}SOD] \text{ or } [Cu^{+}SOD] = (k_1/k_2)[Cu^{2+}SOD] \quad (9)$$

$$[CuSOD]_{tot} = [Cu^{2+}SOD] + [Cu^{+}SOD] = [Cu^{2+}SOD](1 + k_1/k_2) \quad (10)$$

$$k_{obs}\ (s^{-1}) = \{k_1[Cu^{2+}SOD] + k_2[Cu^{+}SOD]\} = 2k_1[Cu^{2+}SOD] = 2k_1[CuSOD]_{tot}/(1 + k_1/k_2) \quad (11)$$

$$k_{obs}\ (M^{-1}\ s^{-1}) = 2k_1/(1 + k_1/k_2) = 2k_1k_2/(k_1 + k_2) \quad (12)$$

$$\text{if } k_1 = k_2,\ k_{obs}\ (M^{-1}\ s^{-1}) = k_1 \quad (13)$$

$$\text{if } k_1 > k_2,\ k_{obs}\ (M^{-1}\ s^{-1}) = 2k_2 \quad (14)$$

All rate constants are given for enzyme concentration, not copper concentration. Each CuZnSOD binds two copper ions, one per subunit.

Results

The specific activities of superoxide dismutation catalyzed by bovine CuZnSOD and Cu-apoSOD are plotted as a function of pH in Figure 1. The pH profiles are distinct for the two different forms of the enzyme. As reported previously, CuZnSOD catalyzes the disproportionation of superoxide in a manner that is relatively pH independent from pH 5 to pH 10 (Figure 1A). As has been noted previously,[16,18,22−24] the activity of Cu-apoSOD, the zinc-deficient form of the enzyme, is pH-dependent (Figure 1B). In order to ascertain if the drop in rate was due to dissociation of Cu^{2+} from Cu-apoSOD, the effect of adding the metal ion chelator EDTA was investigated (Figure 1C). Comparison of parts B and C of Figure 1 indicates that the activity of Cu-apoSOD is independent of metal chelator between pH 5−8, indicating that the presence of EDTA did not cause metal loss. Above pH 8.0, and in the absence of EDTA, the activity of Cu-apoSOD is observed to increase, reaching a maximum at pH 10.2 (Figure 1B). In the presence of EDTA, above pH 8.0, the activity of Cu-apoSOD continues to decrease (Figure 1C). Further evidence against metal ion loss in the pH 5−8 region was our observation that dialysis of either the oxidized or reduced form of Cu-apoSOD, using the same concentrations of enzyme and buffers as in the pulse radiolysis experiments, led to no metal loss at either pH 5 or pH 8, as determined by AA.

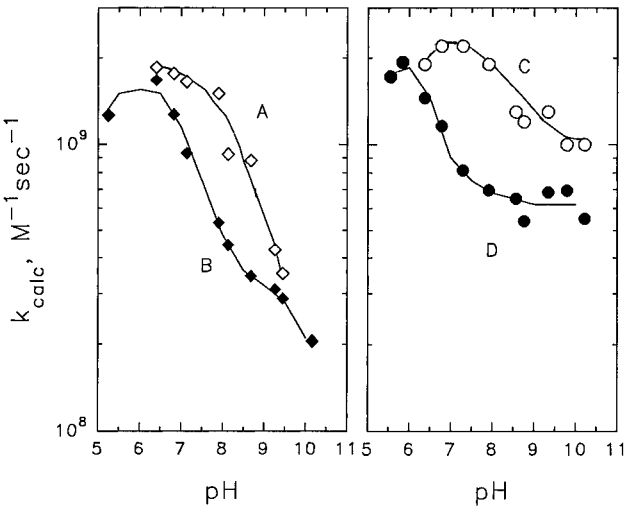


Figure 2. pH dependence of the specific activity of Cu-apoSOD as measured by pulse radiolysis. (A) shows the rate constant for reduction of Cu(II)apoSOD, k_1, and (B) the rate constant during turnover, k_{cat}, both as a function of pH (10 μM Cu-apoSOD, 40 mM formate, 10 mM acetate, no EDTA). (C) and (D) show the analogous results in solutions containing EDTA (25 μM Cu-apoSOD, 9 mM formate, 45 mM phosphate, 100 μM EDTA).

Pulse radiolysis experiments have demonstrated that the two rate constants for the individual steps of the SOD catalytic cycle can be measured independently for CuZnSOD.[3,4] As noted earlier, the rate constant for the reduction step, k_1, is equal to the rate constant for the oxidation step, k_2, at pH values between 5 and 9.5 for CuZnSOD (reactions 1 and 2). In order to determine if the pH-dependent drop in rate for Cu-apoSOD (Figure 1B,C) is due to a specific step in the catalytic cycle, we measured the rate constant k_1 for reduction of Cu^{II}-apoSOD. Excess enzyme relative to superoxide was used in order to prevent turnover. Figure 1D shows that in the pH range 5−8, the rate for the first step of the catalytic cycle, i.e., the rate of reduction of the copper center of Cu^{II}-apoSOD by superoxide is, within experimental error, the same as that of Cu^{II}ZnSOD and is likewise pH-independent.

Experiments to determine k_1 and k_{cat} for Cu-apoSOD as a function of pH were also carried out in the presence and absence of EDTA (parts A, B and C, D of Figure 2, respectively); similar differences between k_1 and k_{cat} between pH 5 and pH 8 were observed.

Discussion

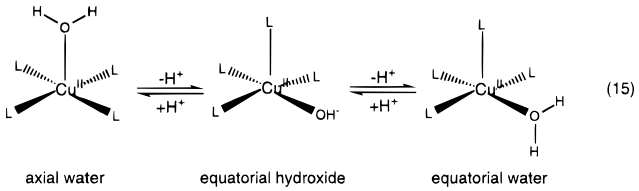
CuZnSOD: pH-Independent Catalytic Properties. Studies using pulse radiolysis have shown that bovine CuZnSOD catalyzes the dismutation of superoxide at very high efficiency, with no change in rate as a function of pH over the range 5−9.5.[3,4] There are two steps to the mechanism, reduction of Cu^{II}ZnSOD by superoxide (reaction 1) followed by reoxidation of Cu^{I}ZnSOD by another superoxide (reaction 2). Both of these reactions occur with the same, nearly diffusion controlled, rate constant, which, in both cases, is invariant with pH over the range 5−9.5. Thus, the rates of the reduction and reoxidation steps are independent of the redox state of the copper, suggesting that it is the charged groups on the exterior of the protein that are the predominant influences on the rates of reaction with the anionic superoxide substrate. Since the rate-limiting step is controlled by diffusion (second-order rate constant 2.4×10^9 $M^{-1}\ s^{-1}$),[4,6] all other processes, such as proton transfer, must occur faster.

CuZnSOD: pH-Independent Spectroscopic Properties. The vis−UV and EPR spectroscopic properties of Cu^{II}ZnSOD

(22) Banci, L.; Bertini, I.; Cabelli, D. E.; Hallewell, R. A.; Tung, J. W.; Viezzoli, M. S. *Eur. J. Biochem.* **1991**, *196*, 123−128.
(23) O'Neil, P.; Fielden, E. M.; Cocco, D.; Calabrese, L.; Rotilio, G. In *Oxy Radicals and Their Scavenger Systems*; Cohen, G., Greenwald, R. A., Eds.; Elsevier Science Publishing Co.: New York, 1983; Vol. I, pp 316−319.
(24) Hirose, J.; Kano, H.; Kidani, Y.; Iwamoto, H.; Hiromi, K. *Chem. Pharm. Bull. (Tokyo)* **1992**, *40*, 506−508.

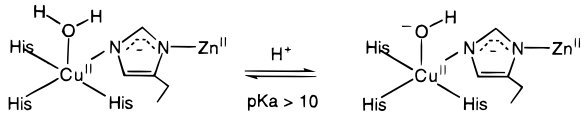
are likewise pH-invariant over a wide range (pH 5−9.5).[3,4,12] The structure of Cu^{II}ZnSOD as determined by X-ray crystallography shows the copper ion to be coordinated by one axially-bound water molecule in addition to three histidyl imidazoles and one imidazolate, which provides the bridge to the zinc ion.[25] Although the copper site is considerably distorted relative to a normal tetragonal Cu^{II} site, the structural and spectroscopic characteristics of the site indicate that it is best described as a distorted tetragonal site with a weakly bound axial water ligand.[26,27] The presence of that water molecule makes the pH independence of the spectroscopic properties of Cu^{II}ZnSOD between pH 5 and pH 9.5 quite remarkable, especially when they are compared to those of other copper proteins and simple coordination complexes that contain water molecules coordinated to Cu^{II}.

Aqueous copper ion is known to have a pK_a of 6.8.[28] Complexes of Cu^{II} with at least one water ligand are generally found to have pK_a values in the range of 7.5−9.0 and several Cu^{II}-containing proteins with bound water are known to convert to the hydroxide form in this same pH range, e.g., copper-substituted carbonic anhydrase, met apo hemocyanin, and laccase.[29−32] One can assume that a low pK_a for a water molecule bound to Cu^{II} indicates that the resulting hydroxide ligand is located in an equatorial rather than an axial position on the copper ion even if the water ligand was initially in an axial position (eq 15). The interaction between the copper ion



(15)

and its axial ligands is much weaker than that with its equatorial ligands,[33] and an abnormally high pK_a, such as is found in Cu^{II}-ZnSOD, is strong evidence that the hydroxide ligand, when it is finally formed at very high pH, remains in an axial position on the Cu^{II} center.



Further evidence of unusually weak hydroxide and other anion binding to Cu(II) in CuZnSOD comes from ^{19}F NMR experiments in which the ^{19}F spin−spin relaxation rate in the presence of the enzyme was found to parallel the enzymatic activity, from which it was concluded that F^- acts as a substrate analog of superoxide.[34,35] The affinity of F^- for bovine SOD was also found to be weak (2 M^{-1}), as expected for a ligand bound in the axial position. The ^{19}F NMR results for CuZnSOD were compared to those found for other copper-containing proteins and low-molecular-weight copper complexes. Interestingly, the ^{19}F relaxation rate of other proteins such as laccase, azurin, and copper-substituted carbonic anhydrase, as well as small copper complexes such as Cu(II)$(DDC)_2$ (DDC = diethyldithiocarbamate), were found to be very low relative to those of CuZnSOD.[36] As described above, most copper proteins and complexes have normal pK_a values for ionization of water coordinated to Cu(II). Thus, the pK_a of the coordinated water and the exchange rate of coordinated fluoride are both anomalously high for CuZnSOD, indicating that both fluoride and hydroxide are weakly bound, presumably in an axial position, to Cu(II) in CuZnSOD.

The unusual ability of Cu(II) in CuZnSOD to restrict anionic ligands such as hydroxide or fluoride to weakly bound axial positions is likely to be due to the presence of the imidazolate bridge, since other copper proteins with imidazole ligands behave in a normal fashion; i.e., they have pK_a values in the range of 7.5−9.0. This conclusion is also supported by our earlier observation that yeast His63AlaSOD,[17] in which the bridging histidyl residue is replaced by alanine, has a pK_a for water coordinated to Cu^{II} that is in the normal range. The implications of this property are addressed in our mechanistic proposal described below.

It is also interesting to note that the Thr137-to-Ile137 mutant human CuZnSOD retains high SOD activity and retains the pH independence of that activity relative to the wild type enzyme, since that mutant CuZnSOD has been shown to have no water ligand coordinated to the Cu^{II} ion.[37] Thus, we can conclude that either a weakly bound axial water ligand with a high pK_a or no water ligand at all on the Cu^{II} ion is compatible with high, pH independent SOD activity and that a strongly bonded water ligand with a low pK_a leads to pH-dependent SOD activity.

Cu-apoSOD: pH-Dependent Catalytic and Spectroscopic Properties. The spectroscopic properties of bovine Cu^{II}-apoSOD were reported in 1979 to be pH-dependent due to migration of Cu^{2+} from the native copper site of one subunit to the native zinc site of another to form subunits containing Cu^{II} in both sites.[19] Earlier work and subsequent studies demonstrated that the SOD activity of bovine Cu-apoSOD was likewise pH-dependent.[16,18,22,23] At that time, it was concluded that the pH-dependent activity of Cu^{II}-apoSOD reported in these studies was due to pH-dependent rearrangement of the Cu^{2+} ions.[18] However, reexamination of the published data indicates that the observed pH dependence cannot be due solely to the copper migration since the SOD activity is pH-dependent in a range of pH well below that at which appreciable copper migration occurs, i.e., pH < 8.

The activity profile of the zinc-deficient form of superoxide dismutase is pH dependent (Figures 1B,C and 2B,D). Below pH 8, the curves are all similar and demonstrate a marked dependence of the activity on pH. Above pH 8.0, and in the absence of EDTA, the activity of Cu-apoSOD is observed to increase, reaching a maximum at pH 10.2 (Figure 1B). This behavior is directly attributable to metal migration (see below).

(25) Tainer, J. A.; Getzoff, E. D.; Beem, K. M.; Richardson, J. S.; Richardson, D. C. *J. Mol. Biol.* **1982**, *160*, 181−217.
(26) Bertini, I.; Banci, L.; Piccioli, M. *Coord. Chem. Rev.* **1990**, *100*, 67−103.
(27) Valentine, J. S.; Pantoliano, M. W. In *Copper Proteins*; Spiro, T. G., Ed.; John Wiley and Sons, Inc.: New York, 1981; pp 292−358.
(28) Basolo, F.; Pearson, R. G. In *Mechanisms of Inorganic Reactions, A Study of Metal Complexes in Solution*, 2nd ed.; Wiley: New York, 1967; p 32.
(29) Allison, J. W.; Angelici, R. J. *Inorg. Chem.* **1971**, *10*, 2233−2238.
(30) Himmelwright, R. S.; Eickman, N. C.; Solomon, E. I. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 243−247.
(31) Tamilarasan, R.; McMillin, D. R. *Biochem. J.* **1989**, *263*, 425−429.
(32) Taylor, J. S.; Coleman, J. E. *J. Biol. Chem.* **1971**, *246*, 7058−7067.
(33) Cotton, F. A.; Wilkinson, G. In *Advanced Inorganic Chemistry*, 5th ed.; Wiley: New York, 1988; pp 766−774.
(34) Viglino, P.; Rigo, A.; Stevanato, R.; Ranicri, G. A. *J. Magn. Reson.* **1974**, *34*, 265−274.
(35) Viglino, P.; Rigo, A.; Argese, E.; Calabrese, L.; Cocco, D.; Rotilio, G. *Biochem. Biophys. Res. Commun.* **1981**, *100*, 125−130.
(36) Rigo, A.; Viglino, P.; Rotilio, G. In *Handbook of Methods for Oxygen Radical Research*; Greenwald, R. A., Eds.; CRC Press: Boca Raton, FL, 1985; pp 227−232.
(37) Banci, L.; Bertini, I.; Cabelli, D. E.; Hallewell, R. A.; Luchinat, C.; Viezzoli, M. S. *Inorg. Chem.* **1990**, *29*, 2398−2403.

As shown in Figures 1B,C and 2B,D, the catalytic rate constant, k_{cat}, for the dismutation reaction is essentially independent of the presence of metal chelator in the lower pH range, i.e., pH 5.0−8.0. The activity of this protein decreases markedly despite the fact that the Cu^{II} geometry appears to be unaltered between pH 5 and pH 8.0, as judged by the spectroscopic properties.[19] This decrease in activity as a function of pH is in sharp contrast to the pH independent activity of CuZnSOD in this pH range, indicating that the Zn^{2+} in the zinc site and the bridging imidazolate have a profound effect in determining the pH independent activity of this enzyme. In the case of CuZnSOD, the rate constants for the individual steps of the reaction were found to be identical, i.e., $k_1 = k_2 = 2 \times 10^9$ M^{-1} s^{-1} and pH-independent over the pH range 5−9.5.[3,4] At issue, therefore, was whether the decrease in activity of the Cu-apoSOD as a function of pH could be attributed to one specific step in the catalytic redox cycle, i.e., the reduction or oxidation step. We, therefore, determined directly the rate of reduction of Cu^{II}-apoSOD in a single turnover experiment (i.e., [Cu^{II}-apoSOD] ≫ [O_2^-]). We found the rate to be the same, within experimental error, as that found for CuZnSOD in the lower pH range, i.e., between pH 5.0 and pH 8.0 (Figure 1D).[38] This result implies that the decrease in activity in this pH range can be attributed to a process that occurs in the second step of the mechanism, i.e., reoxidation of the copper center and subsequent release of hydrogen peroxide. The implications of this finding will be discussed in our mechanistic proposal described below.

The activity profile of the zinc-deficient form of superoxide dismutase has been reported earlier, and the pH-dependent behavior above pH 8.0 has been attributed to metal migration.[18] Metal migration was originally discovered by monitoring the EPR and visible absorption spectra of Cu-apoSOD as a function of pH at very high enzyme concentration (1 mM).[19] Metal migration yields a protein in which half the copper in the catalytically active site of the protein can now be found in another site, i.e., the zinc site. The enzymatic activity for Cu-apoSOD shown in Figure 1B approaches that found for CuZnSOD (Figure 1A) at high pH as the copper in Cu-apoSOD migrates into the zinc site. Thus, putting copper in the empty zinc site causes an increase in SOD activity. However, inspection of Figure 1B indicates that metal migration during our pulse radiolysis experiments occurs with a higher apparent pK_a, i.e., pK_a > 10, rather than that found in the prior spectroscopic studies, i.e., pK_a = 8.2, which were carried out at much higher enzyme concentrations.[19] We account for this apparent discrepancy by noting that the metal migration occurs either from one protein molecule to another or from one subunit to another, and therefore the rate and the extent of the migration are likely to be dependent on the protein concentration. Thus, we expect the extent of metal migration to increase as the concentration of Cu-apoSOD increases. In support of this prediction is the observation that the rate constant drops almost linearly until pH ∼9 when the enzyme concentration is 1 μM (Figure 1B) but starts to level off at pH ∼8 when the enzyme concentration is 10 μM (Figure 2B,D). We attribute the leveling off to the increasing contribution of CuCuSOD subunits formed as a result of metal migration from copper to zinc sites.

Above pH 8, the rate of superoxide dismutation by Cu-apoSOD is sensitive to the presence of EDTA (Figure 1C). Metal migration occurs from one protein molecule to another or from one subunit to another, and therefore it is not surprising that this process is affected by the presence of a metal chelator.

Mechanistic Proposal. The overall rate constant for the noncatalyzed spontaneous dismutation reaction of superoxide radicals into dioxygen and hydrogen peroxide is pH-dependent. The maximum in the rate occurs where the pH equals the pK_a, i.e., the pH at [O_2^-] = [HO_2], since O_2^- reacts very rapidly with HO_2 to give O_2 and HO_2^-.[39] Catalysis of superoxide disproportionation by metal complexes is often pH-dependent; the reasons for this are related to pH-dependent changes in coordination geometry or mechanistic pathway (outer-sphere vs inner-sphere mechanism).[40,41]

The catalytic rate constants for CuZnSOD are pH-independent, in striking contrast to most metal complex catalysts of this reaction. We have shown here that the Cu-apoSOD form of the enzyme catalyzes the disproportion of superoxide via a pH-dependent process. In addition, our previous work on yeast H63ASOD[17] also indicates that removal of the bridging imidazolate results in a enzyme with pH dependent spectroscopic properties and activity. During the last 30 years, the functional significance of this bridge has intrigued researchers who have implicated it in both mechanistic and structural roles. Here we have shown that the bridging imidazolate moiety is required in the second step of the catalytic cycle, i.e., reoxidation of the copper center of SOD and subsequent formation of hydrogen peroxide. The question that remains is: How does the bridging zinc−imidazolate moiety contribute to this process?

There are several possible steps in the catalytic mechanism that might lead to a pH dependence. The first of these, ionization of water on Cu^{II} leading to inhibition by bound hydroxide, is a plausible mechanism for inhibition of the catalytic mechanism, i.e., reduction of Cu^{II} by superoxide. This possibility does not correlate with the low pK_a of the SOD activity. As shown in our earlier work, both yeast H63ASOD and Cu-apoSOD[17] undergo a pH-dependent spectroscopic transition with a pK_a = 9.1. This transition is characteristic of ionizing water bound to Cu^{II}-containing complexes and proteins. Further, we have shown that the rate for reduction of Cu-apoSOD is the same as that found for CuZnSOD, and thus hydroxide does not inhibit the reduction step. A second possible mechanism, oxidation of Cu^{I} by HO_2 rather than O_2^- in the second step of the catalytic mechanism, is also feasible, but the pH dependence of the catalytic rate does not match that expected for protonation of O_2^-. A third possibility is that the electrostatic potential is altered for the Cu-apoSOD form of the enzyme. However, such an alteration would be expected to affect both the reduction and oxidation steps of the reaction, and this is not consistent with our data. The fourth possibility, and the one that we favor, is that the pH dependence of the catalytic rate is caused by slow product release, i.e., that the dissociation of the peroxide formed in the second step of the mechanism from the Cu^{II} center becomes rate-limiting in Cu-apoSOD.

Why would the dissociation of peroxide from Cu^{II} in the absence of the zinc−imidazolate moiety in Cu-apoSOD be slower than in wild type CuZnSOD? We know that the pK_a of water bound to Cu^{II} in wild type CuZnSOD is abnormally high. We conclude, as described above, that this is due to the fact that the hydroxide ligand, when it is formed by deprotonation

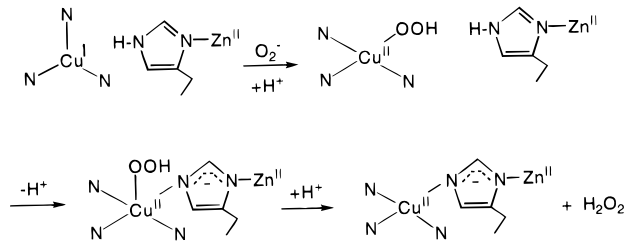
(38) The decrease in rate above pH 9.0 is expected due to metal migration. Copper(II) in the copper site of SOD has a maximum absorbance at 680 nm while copper(II) in the zinc site has a maximum absorbance at 820 nm. Measurement of the reduction rate was carried out by monitoring the copper absorbance at 680 nm.

(39) Bielski, B. H. J.; Cabelli, D. E. *Int. J. Radiat. Biol.* **1991**, *59*, 291−319.

(40) Felix, K.; Lengfelder, E.; Deters, D.; Weser, U. *Biometals* **1993**, *6*, 11−15.

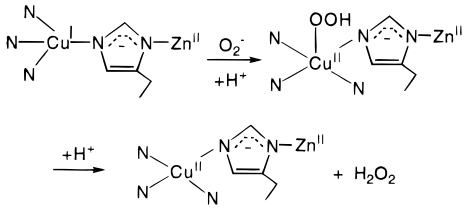
(41) Cabelli, D. E.; Bielski, B. H. J.; Holcman, J. *J. Am. Chem. Soc.* **1987**, *109*, 3665−3669; Weinstein, J.; Bielski, B. H. J. *J. Am. Chem. Soc.* **1980**, 102, 4916−4919; Klug-Roth, D.; Rabani, J. *J. Phys. Chem.* **1976**, 80, 588−591.

Scheme 1



of the bound water at high pH, cannot move into a more strongly bonded, equatorial position in the Cu^{II} coordination sphere. In Cu-apoSOD, the pK_a of the water ligand to the Cu^{II} is normal, and we therefore conclude that the hydroxide ligand can move into an equatorial position, just as cyanide does when it binds to the wild type protein.[42] It therefore appears likely that peroxide, O_2^{2-}, or more likely hydroperoxide, HO_2^-, binds in an equatorial position in Cu-apoSOD but, in wild type CuZnSOD, is restricted to a more weakly binding, rapidly-exchanging axial position. The peroxide dissociation step for wild type CuZnSOD is illustrated in Scheme 1.

The source of the proton(s) that protonate(s) peroxide in the catalytic mechanism of CuZnSOD has been the subject of some interest. Reduction of the oxidized protein has been shown to be accompanied by the uptake of one proton per subunit. That proton is believed to protonate the bridging imidazolate in association with the breaking of the bridge upon reduction of the copper. The same proton is thus an attractive possibility for protonation of peroxide as it is formed in the enzymatic mechanism. Attractive as this mechanism appears, there are some problems with it. For example, it has been pointed out by Fee and co-workers that, at higher concentrations of superoxide, the turnover of the enzyme is too fast for this protonation and deprotonation cycle of the bridging histidine to be occurring.[16] It therefore appears that the catalysis under such conditions may proceed without breaking of the imidazolate bridge. Further, a recent X-ray structure of the reduced form of the bovine enzyme shows that the imidazolate bridge remains intact in the crystal.[43,44] Thus, it may be possible that under conditions such as high superoxide concentration the enzyme turns over with the imidazolate bridge remaining intact.



It is our conclusion that the zinc−imidazolate moiety plays a role in the catalytic mechanism of CuZnSOD by ensuring that peroxide leaves rapidly from the coordination sphere of the Cu^{II} ion in the final step of the mechanism. It does so by an internal displacement reaction in which the zinc−imidazolate rebinds to Cu^{II}, forcing the peroxide ligand into an axial position, thus ensuring that it will leave rapidly from the coordination sphere of the copper ion (see Scheme 1). In the bovine Cu-apoSOD, and possibly in the yeast H63ASOD, which lack the zinc−imidazolate moiety, the rate is presumably pH-dependent due to a requirement that the peroxide ligand be protonated to make it a better leaving group so that it can leave more rapidly from the equatorial position of the Cu^{II} ion. The pH dependence of the SOD activities of the bovine Cu-apoSOD as well as yeast H63ASOD suggests that both the zinc and the bridging imidazolate are required to maintain a copper(II) coordination sphere configuration that will give a pH independent SOD activity and therefore a very high SOD activity at physiological pH.

Acknowledgment. This work is supported by grants from the National Institutes of Health (GM28222 to J.S.V.) and the American Cancer Society (PF-3680 to L.M.E.). Pulse radiolysis studies were carried out at Brookhaven National Laboratory under Contract DE-AC02-76CH000016 with the U.S. Department of Energy and supported by its Division of Chemical Sciences, Office of Basic Energy Sciences. We also thank Dr. James A. Roe for helpful discussions of this work.

JA953845X

(42) Paci, M.; Desideri, A.; Rotilio, G. *J. Biol. Chem.* **1988**, *263*, 162−166.

(43) Banci, L.; Bertini, I.; Bruni, B.; Carloni, P.; Luchinat, C.; Mangani, S.; Orioli, P. L.; Piccioli, M.; Ripniewski, W.; Wilson, K. S. *Biochem. Biophys. Res. Commun.* **1994**, *202*, 1088−95.

(44) Rypniewski, W. R.; Mangani, S.; Bruni, B.; Orioli, P. L.; Casati, M.; Wilson, K. S. *J. Mol. Biol.* **1995**, *251*, 282−296.