5 by a 9:1 ratio, ylide equilibration does not lay an important role in desilylation experiments.

Nitrogen Ylides. Desilylation of salts $Me_3SiCH_2N^+R_3$ under the usual conditions (CsF, CH_3CN) is unsatisfactory because the major product is the parent methylammonium salt $CH_3N^+R_3$. The undesired protiodesilylation is due to proton transfer from acetonitrile and can be avoided by using diglyme as solvent. Two representative cases of nonstabilized ammonium ylide trapping have been demonstrated (Table II): entry 1, fragmentation to an alkene; entry 2, rearrangement by 2,3-sigmatropic shift. Previously, such reactions have been possible only under strongly basic conditions ($R'Li + CH_3N^+R_3$).[8]

Desilylation is especially useful for generation of nonstabilized azomethine ylides which can be trapped by 1,3-dipolar addition.[9,10] Best results are achieved by a one-pot procedure where the imine is alkylated with **1** in acetonitrile and the resulting solution of moisture-sensitive immonium salt is immediately treated with CsF and a dipolarophile. This method allows efficient trapping of azomethines even in cases where deprotonation of the ammonium salt might result in an enamine (entries 3, 4, Table II). Analogous azomethine ylides lacking α substituents to stabilize the carbanionic center (α-carbonyl, cyano, phenyl, etc.) are virtually unknown in the literature.[9a,b] Stabilized azomethine ylides are of course much more common and their properties are well understood from the extensive studies by Huisgen[10] and others.[9]

Phosphorus Ylides. Trialkylphosphonium ylide generation from reaction of α-silylphosphonium salts with alkoxide ion has been demonstrated by Schmidbaur et al.[11] Desilylation by chloride ion has also been demonstrated at elevated temperatures, but fluoride ion is reported to give low yields of Wittig product owing to competing protiodesilylation.[12] We have observed no such complications when the CsF/CH_3CN desilylation method is used. Thus, triphenylphosphine reacts readily with **1** to give a crystalline salt (exothermic). Upon treatment with CsF/CH_3CN in the presence of 4-phenylcyclohexanone (20 °C), the salt undergoes desilylation and in situ Wittig reaction to give 4-phenylmethylenecyclohexane (70% yield).

In summary, CsF-induced desilylation provides access to reactive methylides in synthetically useful yield. The reaction is most important in cases where the molecule contains base-sensitive groups or acidic C–H bonds, substituents which preclude ylide generation by strong base deprotonation of methylsulfonium, methylammonium, or methylimmonium salts. General access to nonstabilized azomethine ylides is possible for the first time, and allows synthesis of 3-pyrrolines of interest in natural products chemistry. Work is continuing in this latter area.

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(4) Trimethylsilylmethanol (1.04 g) was added over 5 min to the complex from pyridine (0.79 g) and trifluoromethanesulfonic anhydride (2.82 g) in methylene chloride (20 mL) at −20 °C. The cooling bath was removed, and stirring was continued for 2 h at 20 °C. Pentane (40 mL) was added, salts were removed by filtration, and the pentane solution was passed through a plug of silica gel (~15 g) with more pentane. Evaporation (0 °C, aspirator) gave **1**, 1.8 g as a colorless oil (76%): NMR ($CDCl_3$) δ 0.19 (s, 9 H), 4.27 (s, 2 H). The crude triflate is sufficiently pure for all of the experiments described in Tables I and II. If desired, **1** can be distilled without significant decomposition (bp 156–158 °C (1 atm)). The substance is stable to storage (refrigerator) and is not a potent lachrymator. However, precautions in handling this reactive alkylating agent are recommended.

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E. Vedejs,* G. R. Martinez

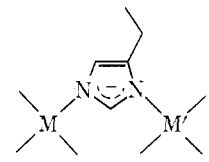
McElvain Laboratory of Organic Chemistry
Chemistry Department, University of Wisconsin
Madison, Wisconsin 53706

Received May 10, 1979

Reversible Loss of Metal Ions from the Zinc Binding Site of Copper–Zinc Superoxide Dismutase. The Low pH Transition

Sir:

Copper–zinc superoxide dismutase, Cu_2Zn_2SOD,[1] from bovine erythrocytes consists of two identical subunits, each of which contains approximately one Cu(II) and one Zn(II)[2] bridged by an imidazolate anion derived from the side chain of histidine 61.[3-11] A number of metal-substituted derivatives have been prepared and spectroscopic studies have convincingly established the location of the metal ions in each derivative.[11-13] Solutions of Cu_2Zn_2SOD or Cu_2Co_2SOD have been



$M = Cu^{2+}$; $M' = Zn^{2+}$, Cu^{2+}, Co^{2+}, Hg^{2+}, Cd^{2+}
$M = Ag^+$; $M' = Cu^{2+}$, Co^{2+}

observed to undergo reversible spectral changes below pH 4.5 attributed to a breaking of the imidazolate bridge by protonation of histidine 61.[10,14-16] In the course of our studies of the metal binding properties of this protein,[17,18] we were struck by the resemblance between the spectra of Cu_2Zn_2SOD, Cu_2Cu_2SOD, and Cu_2E_2SOD[1] below pH 4. We have therefore investigated the behavior of these derivatives at low pH and have reexamined the published data concerning the low pH transition in Cu_2Co_2SOD. It is our conclusion that the metal ion affinity of the native zinc binding site drops abruptly but reversibly at low pH and that the metal ion is consequently

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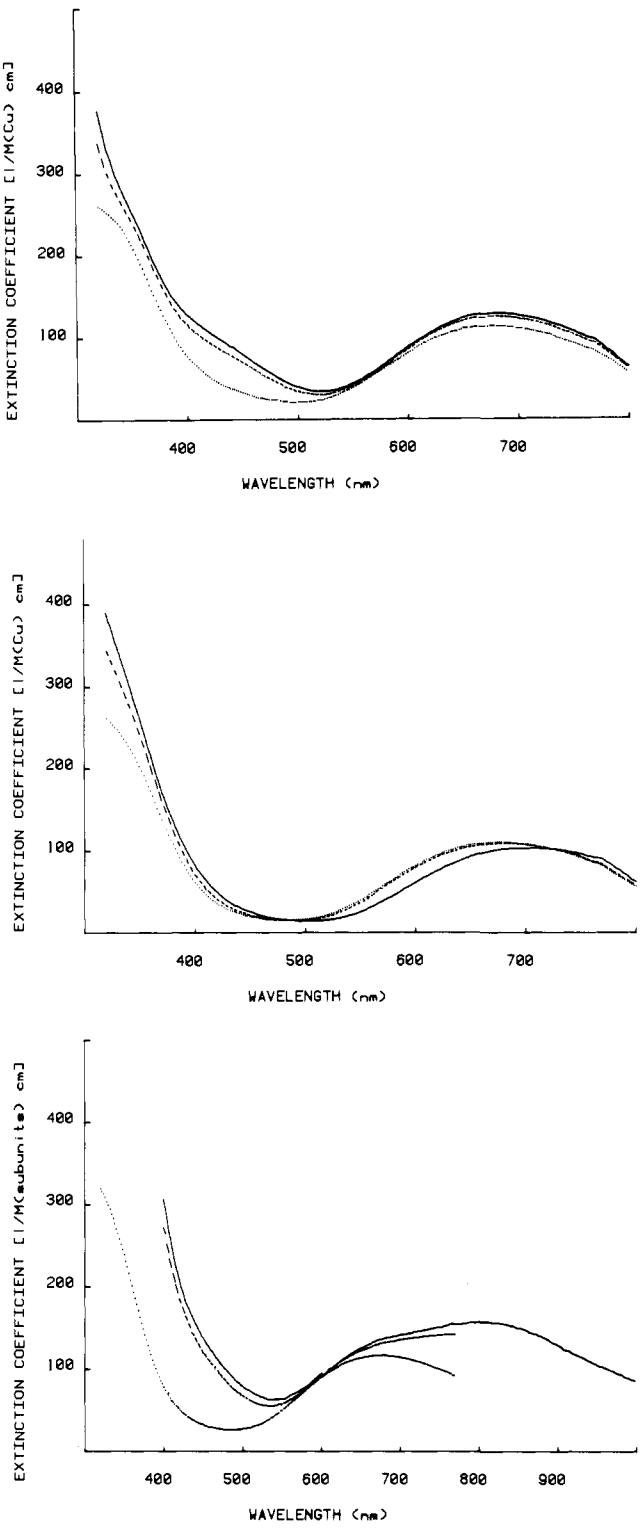

Figure 1. The pH dependence of the visible spectra of Cu_2Zn_2SOD and derivatives. Top panel: Cu_2Zn_2SOD; microcuvette contained 0.7 mL of a 2.06 mM (subunits) solution in 0.1 M potassium phosphate initially at pH 6.1; (--) pH 6.1, (- - -) pH 3.9, (• • •) pH 3.6. Middle panel: Cu_2E_2SOD; microcuvette contained 0.7 mL of a 2.02 mM (subunits) solution in 0.1 M potassium phosphate; (—) pH 5.9, (- - -) pH 4.2, (• • •) pH 3.8. Bottom panel: Cu_2Cu_2SOD; cuvette contained 3 mL of a 0.42 mM (subunits) solution in water at pH 6.1; (—) pH 6.1, (- - -) pH 4.3, (• • •) pH 3.1. Each protein solution was initially filtered through a 0.22-μm-pore-size Millipore filter and centrifuged after pH adjustments to diminish light scattering. The pH was adjusted downward with 1.0 N H_3PO_4 ~1 h before each pH and spectral determination. The extinction coefficients for Cu_2Zn_2SOD and Cu_2E_2SOD are based on the amount of copper determined by atomic absorption photometry. Some inaccuracy in the extinction coefficients (<15%) may have been introduced by spectral distortions from the microcuvettes.

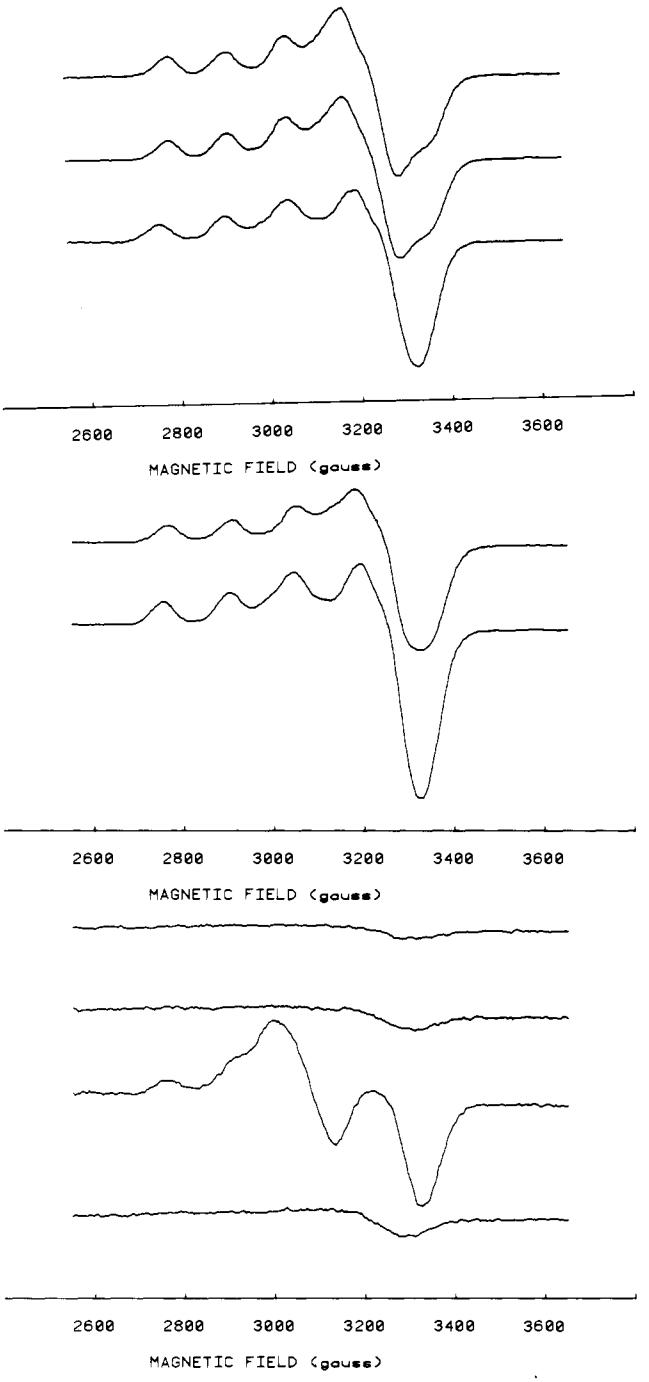

Figure 2. The pH dependence of the ESR spectra[24] of Cu_2Zn_2SOD and derivatives at 30 ± 2 °C. These are the same solutions as those in Figure 1. Top panel: Cu_2Zn_2SOD; the pH values from top to bottom are 6.1, 4.2, and 3.6. The spectral parameters[24] are (top) $A_\parallel$ = 129 G and $g_\parallel$ = 2.261 and (bottom) $A_\parallel$ = 148 G and $g_\parallel$ = 2.263. Middle panel: Cu_2E_2SOD; top spectrum, pH 5.9, $A_\parallel$ = 142 G and $g_\parallel$ = 2.264; bottom spectrum, pH 3.5, $A_\parallel$ = 148 G and $g_\parallel$ = 2.262. Bottom panel: Cu_2Cu_2SOD; the pH values from top to bottom are 6.0, 4.7, 3.3, and 5.4 (readjusted up from pH 3.1 using 1.0 N NaOH).

released into solution. These results are the subject of this communication.

Lowering the pH of solutions of Cu_2Zn_2SOD causes the spectral changes shown in Figures 1 and 2. Equilibrium is reached slowly ($t_{1/2} \simeq 27$ min, 20 °C) when the pH of a 1.0 mM (subunits) solution is dropped from 4.5 to 3.8. Raising the pH back to 4.5, however, results in a rapid ($t_{1/2} \simeq 15$ s, 20 °C) reversion to the original visible spectrum. The vis-UV and ESR spectra of Cu_2Zn_2SOD and Cu_2E_2SOD are very similar at low pH suggesting that Zn^{2+} is no longer bound to the protein. In

accord with this suggestion we have found that dialysis of a 1.0 mM (subunits) solution of Cu_2Zn_2SOD against 0.1 M potassium phosphate at pH 3.6 resulted in removal of 95% of the Zn and 5% of the Cu (by atomic absorption), although the vis-UV and ESR spectra were only slightly changed. Likewise, addition of 1 equiv of Zn^{2+}/subunit to solutions of Cu_2E_2SOD at pH 3.6 caused only small changes in the vis-UV spectra of the solutions, but returning the pH to 6.0 gave spectra identical with those of Cu_2Zn_2SOD. We conclude from these studies either that Zn^{2+} is not bound to the protein at low pH or that it is bound in such a way that its presence does not affect the spectral properties of the Cu(II) chromophore and it is readily removed by dialysis.

Similar results have been obtained for Cu_2Cu_2SOD (see Figures 1 and 2). The ESR experiment at 30 °C is particularly instructive since Cu_2Cu_2SOD is ESR silent at that temperature under our conditions.[18] At low pH, the coppers are uncoupled and the signal due to Cu_2E_2SOD plus the isotropic signal of free aqueous Cu^{2+} appear. The change is almost fully reversed when the pH is raised.

The published vis-UV and ESR spectra of solutions of Cu_2Co_2SOD at low pH[16] also strongly resemble those of Cu_2E_2SOD and free aqueous Co^{2+}. The disappearance of the visible spectrum due to Co^{2+} in an approximately tetrahedral site suggests that Co^{2+} has moved to an octahedral site, such as in $Co(H_2O)_6^{2+}$, where the extinction coefficient of the visible absorption band is expected to be much lower.[19]

The observations described above strongly suggest that the metal ion bound in the native zinc binding site in Cu_2Zn_2SOD, Cu_2Cu_2SOD, or Cu_2Co_2SOD is released in the range $4.5 > pH > 3.0$ and that the metal ion is rapidly re-bound when the pH is raised. The fact that this release of metal ions from the zinc site occurs at very similar pH values whether Zn^{2+}, Cu^{2+}, or Co^{2+} is bound to that site suggests that this phenomenon is not a simple competition between metal ions and protons for the protein side chain ligands. A simple competition reaction would be expected to show a different dependence on pH for the three metal ions if the ligands were conformationally fixed.[20] This observation combined with the observation that E_2Co_2SOD[21] and the apoprotein[14] undergo spectral changes in the same pH range suggest that a pH-dependent conformational change is occurring, the result of which is a lowering of the metal ion affinity of the native zinc site. It is interesting in this regard to note that the protein side chains that comprise the zinc binding site come from a single loop of the polypeptide chain which has a relatively high number of hydrophilic residues in a nonrepetitive secondary structure.[3,4,22] The pH-dependent changes between pH 3 and 4.5 could be accounted for by a conformational change in this loop alone causing only minor changes at the copper binding site.[23]

The pH-dependent nature of the metal ion affinities explains some of the inconsistencies in the early work on metal ion reconstitution of this protein[5] and preparation of metal-substituted derivatives.[10] It also explains the success of the reconstitution procedure of Beem et al.[11,12] in which either Cu^{2+} or Ag^+ is added to apoprotein at pH 3.8 where it binds predominantly at the copper binding site. Our results indicate that the copper binding site is the only strong binding site at that pH.

It is clear from the results described above and previous studies in our laboratory[18] that the metal binding properties of copper-zinc superoxide dismutase are strongly pH dependent. Future studies will be directed toward elucidating the kinetics and thermodynamics of the metal binding reactions of this protein over a wide range of pH.

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References and Notes

(1) Abbreviations: Cu_2Zn_2SOD, native bovine erythrocyte superoxide dismutase; Cu_2M_2SOD (M = Co, Cu) derivative of the native enzyme in which M(II) has been substituted for Zn(II); Cu_2E_2SOD, derivative in which the zinc site is vacant (E = empty); E_2Co_2SOD, derivative in which the copper site is vacant and Co(II) is bound to the zinc site.
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(23) It should be noted that a reorganization of the ligands that comprise the metal binding region may have occurred at low pH resulting in the existence of only one strong metal binding site, with a higher affinity for Cu^{2+} than for Zn^{2+} or Co^{2+}. In other words, we cannot be certain that the same four histidyl side chains are bound to Cu^{2+} at low pH as are bound at high pH.
(24) ESR spectra were recorded using an aqueous ESR cell (Wilmad) at ambient temperature (30 ± 2 °C) together with a Varian E-12 spectrometer at 100-kHz field modulation and a sweep rate of 180 G/min. The Cu_2Zn_2SOD and Cu_2E_2SOD spectra were obtained using a microwave power of 30 mW at a frequency of 9.41 GHz and a modulation amplitude of 8.0 G. The gain was 2500 while for Cu_2Cu_2SOD it was increased by a factor of 2.5 and the modulation amplitude was increased to 10.0 G. The magnetic field was calibrated using the signal of Mn(II) naturally present as an impurity in SrO.[18] Microwave frequency measurements were made using a Hewlett-Packard Model 5255A frequency meter. All ESR spectral parameters are defined according to B. Malmstrom and A. Vänngård, *J. Mol. Biol.*, **2,** 118 (1960).

Michael W. Pantoliano, Peter J. McDonnell
Joan S. Valentine*
Department of Chemistry, Busch Campus
Rutgers, The State University
New Brunswick, New Jersey 08903
Received June 26, 1979

P-450 Type Oxygen Activation by Porphyrin-Manganese Complex

Sir:

Since cytochrome P-450 was first isolated in 1955,[1] much attention has been given to structural[2] and/or mechanistic[3] elucidation of this interesting and somewhat unique enzyme.

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