Chemistry of the Imidazolate-Bridged Bimetallic Center in the Cu–Zn Superoxide Dismutase and Its Model Compounds

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Ligand-bridged polymetallic centers occur in numerous metalloproteins where they perform electron transfer, oxygen transport, and catalytic redox functions.[1] The properties of a variety of proteins containing such centers are given in Table I.[2-10] One of the most extensively studied members of this group, and probably the best understood, is the Cu–Zn superoxide dismutase (SOD†).

Superoxide dismutases are a ubiquitous group of enzymes. Procaryotes and mitochondria of eucaryotic cells have iron- or manganese-containing superoxide dismutases while a copper- and zinc-containing superoxide dismutase is found in the cytosol of eucaryotic cells.[11-13] These enzymes are believed to protect cells from the toxic effects of superoxide ion, or some product derived from it, by catalyzing the dismutation reaction given in eq 1. Hydrogen peroxide formed in this reaction is destroyed in vivo, for example, by catalase.

$$2O_2^- + 2H^+ = O_2 + H_2O_2 \qquad (1)$$

Bovine erythrocyte SOD has a molecular weight of 31 200 and is composed of two identical subunits.[14,15] Each subunit contains an active site having one copper and one zinc atom. The X-ray crystal structure at 3-Å resolution has been published,[2] and coordinates from 2-Å data are available.[16] As shown in Figure 1, the copper is coordinated to four imidazole nitrogen atoms from histidines-44, -46, -61, and -118 while the zinc is coordinated to histidines-61, -69, and -78 and the carboxylate group of aspartic acid-81. The most interesting and still unique feature of the active site structure is the imidazolate bridge between the two metal ions. The pyrrole hydrogen of the imidazole group of histidine-61 has been removed, allowing for coordination of both copper and zinc. The geometry aground the zinc is approximately tetrahedral, and the four protein ligands to the copper are distorted square planar. Proton NMR data indicate that a water molecule is coordinated to the copper in an axial position.[17,18] The two imidazole groups of the tripeptide His-44-Val-45-His-46 occupy trans positions in the copper coordination sphere. Valine-45 thus blocks access to a second axial coordination site, preventing the copper from becoming six-coordinate.

The copper and zinc can be removed from the enzyme by dialysis against EDTA at low pH,[19] resulting in complete loss of catalytic activity. The apoprotein can be reconstituted with copper and zinc[20] or, alter-

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† Abbreviations: SOD, copper–zinc superoxide dismutase; Cu_2Zn_2SOD, native superoxide dismutase having copper and zinc in their normal binding sites. Other derivatives of SOD are indicated as X_2Y_2SOD, meaning metal X is in the normal copper sites and metal Y is in the normal zinc sites. When Y = E the zinc sites are empty. EDTA, ethylenediamminetetraacetate; ESR, electron spin resonance; DEP, diethyl pyrocarbonate; imH, imidazole; imH_2^+, imidazolium; TSP, 3-(trimethylsilyl)propanesulfonic acid.

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Table I
Proteins Having Ligand-Bridged Polymetallic Centers

protein	function	metal ions[a]	bridging ligand	ref
superoxide dismutase	dismutation of O_2^-	Cu, Zn	histidine imidazolate	2
hemocyanin	O_2 transport	Cu	uncertain	3
tyrosinase	monooxygenase, oxidase	Cu	uncertain	4
blue oxidases[b]	oxidase	Cu	uncertain	5
hemerythrin	O_2 transport	Fe	O, RCO_2^-	6
iron-sulfur proteins[c]	electron transport	Fe	S^{2-}	7
nitrogenase	N_2 reduction	Fe, Mo	S^{2-}	8
cytochrome oxidase[d]	oxidase	Cu, Fe(heme)	RS^- (?)	9, 10

[a] In the polymetallic center. [b] Includes ascorbate oxidase, ceruloplasmin, and laccase. These proteins also contain types 1 and 2 copper. [c] Includes proteins having one, two, or four Fe atoms per active site. A number of flavoprotein dehydrogenases contain Fe/S centers, including NADH and succinate dehydrogenase. [d] This enzyme also contains an additional heme and copper per active site.

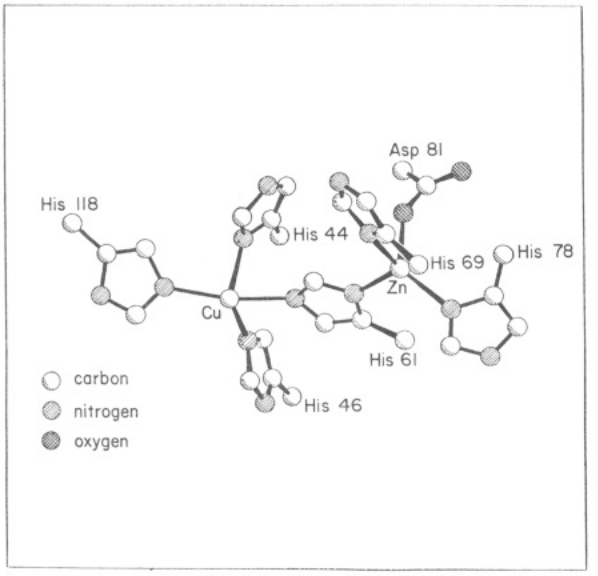

Figure 1. The active site structure of SOD at 2 Å resolution drawn from the published coordinates.[16b] The protein ligands to the two metal ions are shown. Hydrogen atoms have been omitted.

natively, with a variety of other metals. Zinc can be replaced by copper, cobalt, mercury, or cadmium,[20,21] and copper can be replaced by cobalt, silver, or zinc.[22-24] The derivative having copper in both metal binding sites, Cu_2Cu_2SOD, is particularly interesting because the two copper ions are antiferromagnetically coupled through the bridging imidazolate group. The exchange coupling constant, J, was estimated to be -26 cm^{-1} from the temperature dependence of the ESR signal intensity.[21] The specific activity, on a protein basis, of Cu_2Cu_2SOD is the same as that of Cu_2Zn_2SOD.

A detailed mechanism for SOD, proposed independently by two research groups,[12,25] is shown in Figure 2. In this mechanism, reduction of the copper is accompanied by displacement and protonation of the bridging imidazolate group. Reducing the copper results in the uptake of one proton per subunit,[26] which is consistent with the proposed mechanism. Displacement of the bridge also leaves a vacant coordination site on Cu(I),

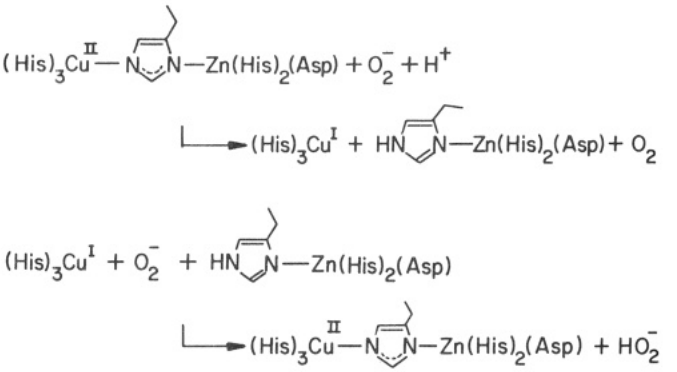

Figure 2. A proposed mechanism for superoxide dismutase. Reproduced from ref 12.

allowing for the possibility of inner-sphere electron transfer in the second step. The proposed role of the zinc is to attenuate the pK_a of the pyrrole nitrogen of histidine-61 so that it will bind Cu(II) instead of a proton but will bind a proton in preference to Cu(I).[12] The bridging ligand may also serve as a proton donor to the developing peroxide dianion in the second step, leading to the production of HO_2^- and re-forming the imidazolate bridge.

The availability of simple inorganic analogues of metalloprotein active sites has led to important insights into their biological chemistry.[27] The synthetic models for hemoglobin and myoglobin[28] and the iron-sulfur proteins[29] provide excellent examples of the usefulness of this approach. At the time when the structure of SOD was first reported, soluble binuclear copper complexes having a bridging imidazolate group had not been synthesized.[30] The preparation and characterization of such molecules were therefore important objectives at the outset of the work described here.

In this Account we discuss synthetic routes to binuclear imidazolate-bridged copper complexes, describe their structures, and compare their chemical and physical properties with those of the enzyme. The decision to prepare complexes having an imidazolate bridge between two copper ions rather than between copper and zinc as in the native enzyme greatly simplified the synthetic problem and was justified by the fact that Cu_2Cu_2SOD has the same catalytic activity as Cu_2Zn_2SOD. We also present studies of superoxide dismutase itself that test various aspects of the pro-

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Table II
Properties of Selected Imidazolate-Bridged Copper(II) Complexes[a]

	$-J$, cm^{-1}	pK_a[b]	ϕ_1,ϕ_2, deg[c]	α_1,α_2, deg[d]	ref
$[Cu_2(bpim)]^{3+}$	81.8		143, 142.2	4.7, 11.4 (estd)	31, 35
$[Cu_2(bpim)(im)]_2^{4+}$	87.6		144.8, 143	4.7, 11.4	31, 35
	35.0		125.5, 130.2	98.2, 80.5	
$[Cu_2(pip)_2(im)]^{3+}$	26.9	6.95	120, 121	88, 88 (estd)	31b
$[Cu_2(TMDT)_2(im)(ClO_4)_2]^+$	25.8	6.95	129, 129	91.8, 90.0	32
$[Cu_2(deim)(PMDT)]^{3+}$	45.2				33
$[Cu_2(PMDT)_2(bzim)]^{3+}$	17.0	5.4	128. 123	96.1, 86.6	35
$[Cu_2(PMDT)_2(2\text{-Mebzim})]^{3+}$	29.8	6.1			35
$[Cu_2(PMDT)_2(2\text{-Meim})]^{3+}$	38.1	7.75	120.6, 120.6	90, 90	35
$[Cu_2(PMDT)_2(biim)]^{2+}$	<0.5				37
$[Cu_2(Gly\text{-}GlyO)_2(im)]^-$	19.1		124.5, 124.1	5.8, 10.4	40
Cu_2Cu_2SOD	26				21

[a] Ligand abbreviations are: bpim, 4,5-bis[[[2-(2-pyridyl)ethyl]imino]methyl]imidazolate; im, imidazolate; pip, 2-[[[2-(2-pyridyl)ethyl]imino]methyl]pyridine; TMDT, 1,1,7,7-tetramethyldiethylenetriamine; deim, 4-[[[2-[[2-(dimethylamino)-ethyl]amino]ethyl]imino]methyl]imidazolate; PMDT, 1,1,4,7,7-pentamethyldiethylenetriamine; biim, 2,2′-biimidazolate; tren, 2,2′,2″-triaminotriethylamine; bzim, benzimidazolate; 2-Meim, 2-methylimidazolate; 2-Mebzim, 2-methylbenzimidazolate; Gly-GlyO, glycylglycinate(2−). [b] pK_a for the reaction $imH_2^+ \rightleftharpoons imH + H^+$ of the bridging ligand. [c] ϕ_1 and ϕ_2 are the Cu-N-C(2) angles (see text). [d] α_1 and α_2 are the dihedral angles between the bridging imidazolate ring and copper coordination planes.

posed catalytic mechanism, especially the role of the zinc and the involvement of the imidazolate bridge.

Simple Models for Cu_2Cu_2SOD

The presence of an imidazolate-bridged bimetallic center in SOD and the suggestion that imidazolate could serve as a bridging ligand in cytochrome oxidase[9] and perhaps other metalloproteins inspired the synthesis of one tetranuclear and numerous binuclear imidazolate-bridged copper complexes by various research groups.[31-40] The coordination geometry of the copper ions and the orientation of the imidazolate ring with respect to the copper coordination plane vary considerably in these compounds. The structures of several of the complexes, determined by single-crystal X-ray diffraction, are shown schematically in Figure 3. Table II gives the spin-exchange coupling constants and geometric features of these complexes and Cu_2Cu_2SOD. Synthetic procedures for all the compounds may be found in the references to Table II.

The value of these compounds as enzyme models is well illustrated by their magnetic properties.[31-33,35-40] All of the complexes exhibit antiferromagnetic exchange interactions with J varying from almost 0 to -87 cm^{-1}. The value of -26 cm^{-1} estimated for Cu_2Cu_2SOD lies within this range and is very close to the results for $[Cu_2(pip)_2(im)]^{3+}$ and $[Cu_2(TMDT)_2(im)(ClO_4)_2]^+$. In no case is the magnetic coupling mediated by the bridging imidazolate group as large as that of the Cu(II)–Fe(III) interaction observed in cytochrome oxidase ($J \leq -200$ cm^{-1}), a result that argues against the proposed imidazolate bridge in that enzyme.[41]

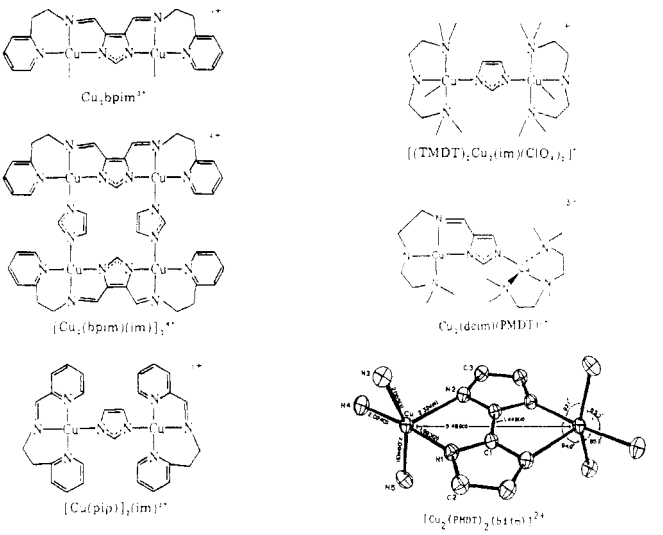
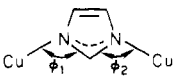


Figure 3. Structures of several imidazolate-bridged copper complexes. Ligand abbreviations are given in Table II, footnote *a*.

The observed antiferromagnetic interaction can in principle occur through either the σ or π system of the bridging imidazolate ligand. A superexchange pathway involving the π system is unlikely because of symmetry mismatch of the relevant ligand orbitals and the copper $d_{x^2-y^2}$ orbitals. The π interaction would be appreciable only if the dihedral angles between the imidazolate ring and copper coordination planes (α_1 and α_2) were considerably different from 0 or 90°. The actual values (Table II) are close to these two limits, making it likely that the σ exchange pathway predominates in these compounds.

It has been suggested that the angles ϕ_1 and ϕ_2 in structure 1 are important in determining the value of



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J for a σ pathway. An increase in these angles would produce stronger coupling since calculations[36] indicate that $|J|$ is a maximum when the Cu–N bonds are par-

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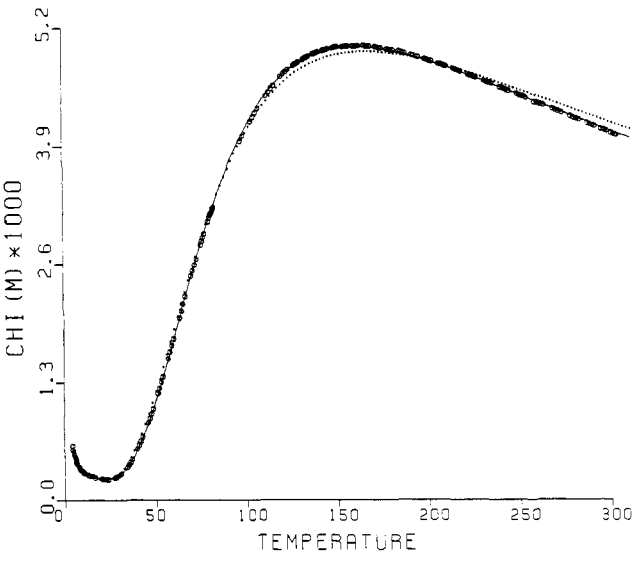

Figure 4. Corrected magnetic susceptibility (cm^3 mol^{-1}) vs. temperature plot for solid $[Cu_2(bpim)(im)]_2(NO_3)_4 \cdot 4H_2O$. Experimental data are shown as open circles. The solid lines shows the best least-squares fit of the data to a model having unequal coupling constants ($J_1 \neq J_2$) for the two types of imidazolate groups while the dotted line was obtained for a model having $J_1 = J_2$ (see ref 35 for discussion).

allel to the imidazolate carbon–carbon bond, $\phi = 144°$. Most of the complexes in Table II have ϕ angles in the range 120–130°. In the one case where the ϕ values are considerably larger, $[Cu_2(bpim)]^{3+}$, the antiferromagnetic coupling is stronger. The overall correlation between J and ϕ is not good, however, indicating that the ϕ angles alone do not determine the magnitude of J.

In an analysis of the variations in J for a series of $[Cu_2(tren)_2X]^{3+}$ complexes, where X = bzim, 2-Meim, and im, it was proposed that the ϕ angles would increase in the order bzim < im < 2-Meim according to the steric requirements of the ring substituents.[36] Such a trend is not seen in the corresponding $[Cu_2(PMDT)_2X]^{3+}$ complexes for which complete structural information is available.[35] Substitution of the imidazolate ring also alters its electronic properties, however, and these electronic factors appear to be of equal or even greater importance than steric effects in determining the magnitude of the exchange interaction. A comparison of the pK_a values of the substituted imidazoles for the reaction $imH_2^+ \rightleftharpoons imH + H^+$ (Table II) indicates that J correlates with the basicity of the bridging ligand.[35] This correlation is reasonable if a σ exchange pathway predominates since substituent effects which alter the strength of the N–H bonds in the imidazolium cation should also influence the σ interaction between the nitrogen lone pair orbitals and the copper $d_{x^2-y^2}$ orbitals. The correlation between J and the pK_a of the bridging imidazole ligand is imperfect, but the differences between the coupling constants and the pK_a's for any two ligands depend only on their substituent differences.

Figure 4 depicts the temperature dependence of the magnetic susceptibility of the $[Cu_2(bpim)(im)]_2^{4+}$ cation.[35] The solid line shows the least-squares fit of the data assuming two different J values and a single g value, which is significantly better than the fit (dotted line) with a single J value. The J values obtained compare favorably with those expected for the two types of imidazolate bridges present in this complex (Figure 3), as exemplified by the bpim and pip or TMDT complexes. The tetranuclear complex is thus an example where two spin-exchange coupling constants between pairs of metal ions in a polymetallic system have been clearly delineated.

ESR spectra of Cu_2Zn_2SOD and Ag_2Cu_2SOD,[22] where the copper is in the normal copper and zinc sites, respectively, indicate that the zinc site copper has a more tetrahedral geometry than the distorted square-planar geometry of the copper site. This result suggests that a better model for Cu_2Cu_2SOD would have one square-planar and one D_{2d} distorted copper ion. The cation $[Cu_2(deim)(PMDT)]^{3+}$ is a step in that direction since, for the first time, the two copper coordination spheres are not identical, although both are expected to be square planar. The J value for this complex falls between those of $[Cu_2(bpim)]^{3+}$ and $[Cu_2(TMDT)_2(im)(ClO_4)_2]^+$, the symmetric complexes having the same coordination spheres as the two halves of $[Cu_2(deim)(PMDT)]^{3+}$.

The presence of a bridging imidazolate ligand does not in itself require an antiferromagnetic interaction, as evidenced by the results for $[Cu_2(PMDT)_2(biim)]^{2+}$.[37] In this cation, each imidazolate group of the biim ligand bridges from an equatorial site on one copper atom to an axial site on the other. The axial ligand on each square-pyramidal copper has little overlap with the $d_{x^2-y^2}$ orbital, which contains the unpaired electron, preventing strong coupling of the spins on the two metal ions.

Imidazolate-bridged binuclear copper complexes have characteristic ESR spectra that arise from the thermally populated triplet state.[42] Figure 5 shows the X-band ESR spectra of a typical imidazolate-bridged model compound, $[Cu_2(TMDT)_2(im)(ClO_4)_2]^+$, and of Cu_2Cu_2SOD in frozen solution. The solution spectrum of $[Cu_2(TMDT)_2(im)(ClO_4)_2]^+$ is the same as that of the solid, indicating that the imidazolate bridge is stable under the solution conditions employed. The spectra are characterized by a broad absorption between 2000 and 4000 G due to the $\Delta M_S = \pm 1$ transition of the triplet state. The sharp feature in the 3200–3300-G region is caused by a small amount of mononuclear copper in the sample. The pronounced signal at ~3600 G is characteristic of the imidazolate-bridged dicopper(II) moiety. The weak signal at 1200–1800 G is the spin-forbidden $\Delta M_S = \pm 2$ transition. In the model compound spectrum this transition consists of seven lines due to the hyperfine interaction with the two copper nuclei ($I = {}^3/_2$). The hyperfine splitting is not resolved in the Cu_2Cu_2SOD spectrum. The excellent agreement between the ESR spectra of the model compounds and Cu_2Cu_2SOD is a strong indication of their structural similarity.

ESR spectroscopy reveals that the bridged cation $[Cu_2(TMDT)_2(im)(ClO_4)_2]^+$ is a major species in solution only over the range $8.5 \leq pH \leq 9.5$.[32] As the pH is lowered, protonation of the imidazolate group first breaks the bridge and then releases imidazolium ion. At still lower pH the TMDT ligand is protonated and free Cu(II) ion is produced. At high pH hydroxide complexes form at the expense of the $Cu_2(im)^{3+}$ unit. Similar results are observed with other imidazolate-

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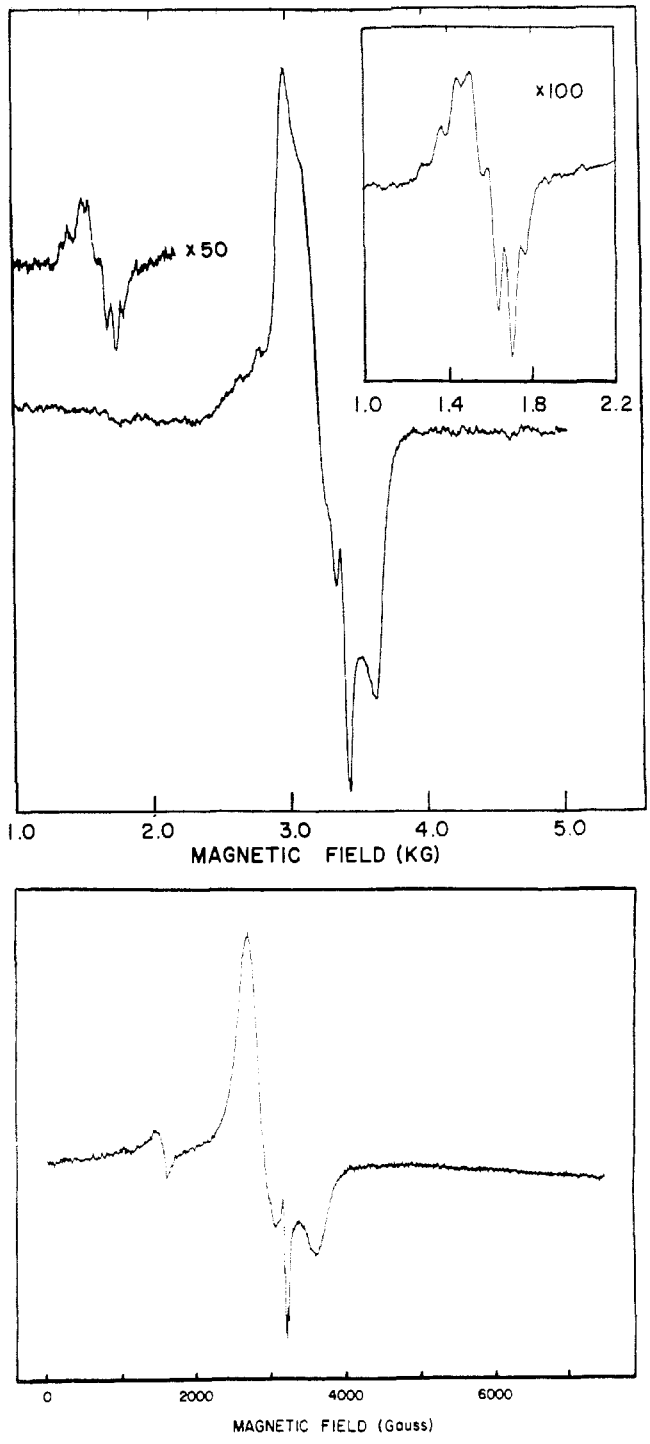


Figure 5. ESR spectra of (a) $[Cu_2(TMDT)_2(im)(ClO_4)_2](ClO_4)$ in 50% aqueous dimethyl sulfoxide at pH 8.5 and 77 K and (b) Cu_2Cu_2SOD at 100 K. The inset in (a) shows the $\Delta M_S = \pm 2$ signal at 50 K and higher gain. Spectra are reproduced from ref 32 for (a) and with permission from ref 21 (copyright 1975, Elsevier (Amsterdam)) for (b).

bridged complexes, as judged by ESR and optical spectroscopy and by potentiometric titrations.[34] In contrast to the simple model compounds, the imidazolate bridge in Cu_2Cu_2SOD is stable over the range $4.5 \leq pH \leq 11$.[43,44] The rather narrow pH range over which the imidazolate bridge is stable in the models restricts their usefulness for solution studies and has led to the development of a second generation of compounds in which the $Cu_2(im)^{3+}$ unit has greater solution stability.

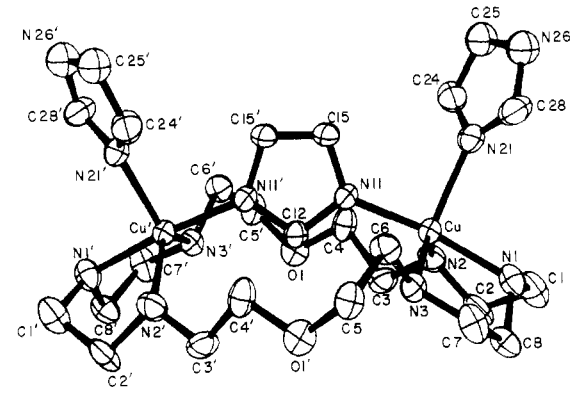
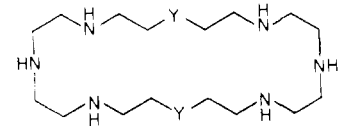


Figure 6. The structure of $[Cu_2(imH)_2(im)\subset A]^{3+}$ showing the 40% probability thermal ellipsoids. Abbreviations: imH, imidazole; A, 1,4,7,13,16,19-hexaaza-10,22-dioxacyclotetracosane. Reproduced from ref 45.

Binucleating Macrocyclic Models for Cu_2Cu_2SOD

Incorporation of the $Cu_2(im)^{3+}$ moiety into the macrocyclic ligand A or A′ results in enhanced stability of



macrocycle A, Y = O
macrocycle A′, Y = CH_2

the bridge in solution.[45,46] The structure of $[Cu_2(imH)_2(im)\subset A]^{3+}$ is shown in Figure 6. Each copper is coordinated to three nitrogen atoms from the macrocycle, the bridging imidazolate, and an additional imidazole ligand, resulting in distorted trigonal-bipyramidal geometry. Although salts of the $[Cu_2(im)\subset A]^{3+}$ and $[Cu_2(im)\subset A']^{3+}$ cations are available, they do not crystallize as readily as the imidazole or *N*-methylimidazole derivatives.[47]

Potentiometric titration of solutions containing $[Cu_2(im)\subset A](ClO_4)_3 \cdot H_2O$ indicated that the imidazolate-bridged species predominates from pH 6 to above pH 10.[46] By comparison, $[Cu_2(TMDT)_2(im)]^{3+}$ is a major species in solution only over the narrow range $8.5 < pH < 9.5$. ESR spectra in the $g = 2$ region of the closely related complex $[Cu_2(im)\subset A']^{3+}$ at various pH values, Figure 7A–E, confirm the presence of the bridged species as the major component in solution from pH 6 to pH 11.5.

Figure 7F–I displays the ESR spectra obtained for various Cu:A′:imH:H^+ ratios. Addition of 1 or 2 equiv of copper per macrocycle in the absence of imidazole results in the appearance of spectra indicating magnetically isolated Cu(II) ions in more than one environment, judging from the number of lines in the $g_\parallel$ spectral region. When imidazole is added to the 1:1 Cu–A′ mixture a dramatic change in the ESR spectrum occurs characteristic of the formation of the imidazo-

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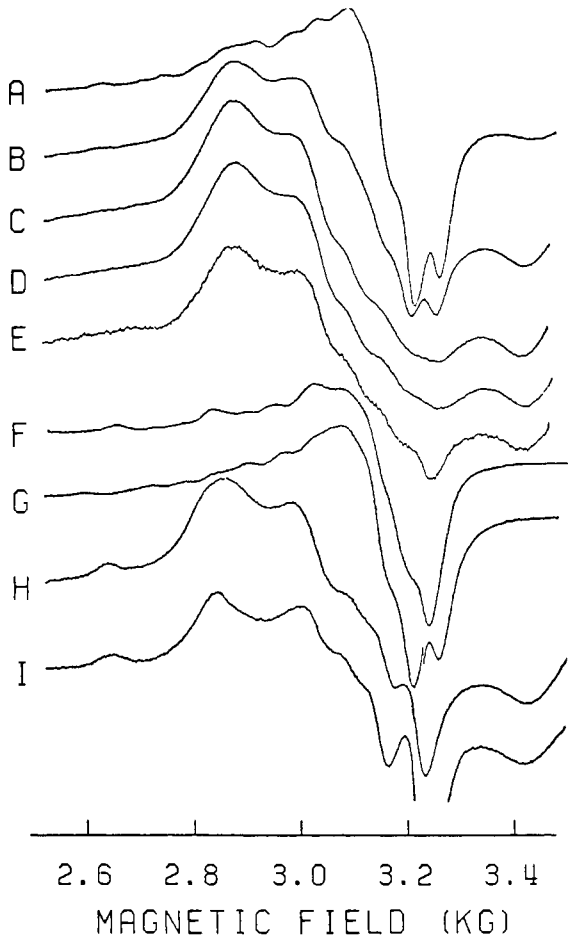

Figure 7. Electron spin resonance spectra of frozen 50% aqueous dimethyl sulfoxide solutions of $[Cu_2(im)\subset A'](ClO_4)_3 \cdot H_2O$ at 115 K as a function of pH [(A) 5.6, (B) 6.2, (C) 7.1, (D) 10.4, (E) 11.5] and at 77 K at various Cu–A′–imH–H^+ ratios [(F) 1:1:0:0, (G) 2:1:0:0, (H) 1:1:1:0, (I) 1:1:1:1]. Reproduced from ref 46.

late-bridged dicopper complex. Since the 1:1 Cu–A′ solution contains very little $[Cu_2\subset A']^{4+}$ (cf. F and G), a redistribution of the copper to form $[Cu_2(im)\subset A']^{3+}$ has occurred. This behavior is similar to the pH-dependent migration of copper that occurs in Cu_2E_2SOD, as discussed below.

Are the complexes that have been synthesized good models for Cu_2Cu_2SOD? The answer seems to be yes. The pH stabilities of the imidazolate bridge in solution for the binucleating macrocyclic complexes and Cu_2-Cu_2SOD are similar, and ESR spectra and J values for all the model compounds are close to those of the protein. These results justify the use of secondary amine nitrogen atoms as ligands in place of the histidine imidazole and aspartate carboxyl groups, which are the ligands in Cu_2Zn_2SOD and presumably also in Cu_2-Cu_2SOD. The question that naturally arises is will these compounds catalyze the dismutation of superoxide ion and, if so, can they be used to investigate the mechanism of the reaction? Although some of the model compounds in Figure 3 have been reported to catalyze the dismutation of superoxide,[48] the significance of this result is questionable. The difficulty with these experiments is that trace amounts of mononuclear copper complexes are very active catalysts. When the dismutase activity of the enzyme is measured, EDTA is added to the buffer to chelate adventitious copper and prevent it from acting as a catalyst.[49] The imidazolate-bridged model compounds all contain traces of mononuclear copper, as judged by ESR spectroscopic and magnetic susceptibility data, and the use of EDTA in assaying their activity is precluded because EDTA readily removes the copper, even from the binucleating macrocycles.[50] The observed catalytic activity therefore could have arisen from the impurities and not from the imidazolate-bridged model compounds.

Superoxide Reactions

Reaction of superoxide ion with Cu(II) or Cu(I) complexes in aprotic media provides a means of exploring features of the proposed mechanism for SOD, such as the formation of copper–superoxide complexes as intermediates in the electron-transfer process. During a study of the reaction between bis(salicylato)copper(II) tetrahydrate and superoxide ion in dry dimethyl sulfoxide, reduction of the copper was found to occur. Further addition of superoxide resulted in an ESR spectrum which was interpreted to result from a "paramagnetic equilibrium complex", $Cu(I)(O_2^-) \rightleftharpoons Cu(II)(O_2^{2-})$.[51] Reinvestigation of this reaction, however, demonstrated that the observed ESR signal arises from Cu(II) complexes of the salicylate dianion and not from a Cu(I)–superoxide complex.[52] Superoxide thus serves as a base in this reaction to deprotonate the phenolic hydroxyl group of salicylate.

A stable Cu(II)–superoxide complex has been prepared in dry dimethyl sulfoxide using the macrocyclic complex Cu(tet b)$^{2+}$ (tet b = *rac*-5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazacyclotetradecane).[53] The stability of this ESR-silent superoxide complex is presumably due to the low redox potential of Cu(tet b)$^{2+}$, which precludes electron transfer to copper.

The Role of Zinc

The role of zinc in the catalytic mechanism of SOD proposed above involves its effect on the pK_a of the pyrrole hydrogen of histidine-61. As we shall see later, this effect is not essential to the enzymatic activity. What other function might the zinc then serve? There is a substantial body of evidence that zinc can play a structural role in proteins,[54] stabilizing the native tertiary and quaternary structures. Various experiments have suggested such a role for the zinc in SOD.

NMR spectra of the exchangeable histidine N–H protons in several forms of SOD are shown in Figure 8.[55] Six of the eight histidines per subunit are in the active site. The native oxidized protein gives a poorly resolved spectrum due to paramagnetic broadening from Cu(II). The native reduced protein has a well-resolved spectrum, however, consisting of at least five peaks. The lowest field peak corresponds to a single proton. Observation of a histidine N–H proton indi-

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(50) The failure of EDTA to remove copper from Cu_2Zn_2SOD or Cu_2Cu_2SOD at neutral pH is presumably a kinetic phenomenon.

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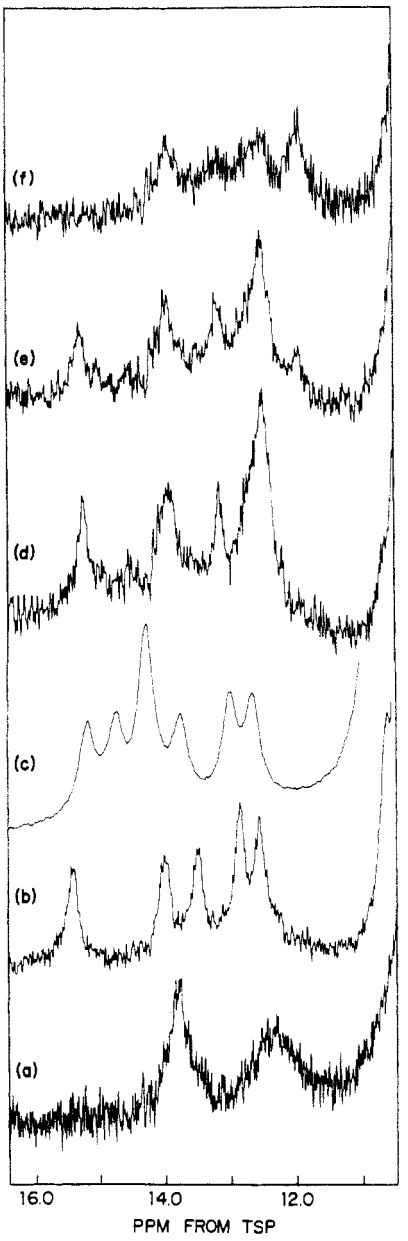

Figure 8. The 220-MHz proton NMR spectra of various forms of bovine erythrocyte SOD in H_2O, pH 6 phosphate buffer, 18 °C. Spectra a, b, d, e, and f were obtained under identical conditions (sweep rate 300 Hz/s, 512 scans except for spectrum a which was scanned 820 times). Spectrum c was scanned 300 times with a sweep rate of 25 Hz/s. (a) Native oxidized; (b) native reduced; (c) apo + 2.0 equiv of Zn^{2+} per subunit; (d) apo + 1.0 equiv of Zn^{2+}; (e) apo + 0.5 equiv of Zn^{2+}; (f) apoprotein. Reproduced from ref 55.

cates that it undergoes exchange with water that is slow on the NMR time scale. The apoprotein NMR spectrum shows only broad, weak signals due to an enhanced rate of exchange in the absence of the metal ions. Addition of one Zn(II) per subunit to the apoprotein results in a dramatic sharpening of the spectrum and an overall appearance not greatly different from that of the native reduced spectrum. This result indicates that zinc alone can organize the active site into a structure similar to that of the native enzyme. Addition of a second equivalent of zinc per subunit produces further changes in the NMR spectrum, indicating additional binding of zinc. Similar conclusions concerning the effect of zinc on the structure of SOD were made from examination of changes in the complete

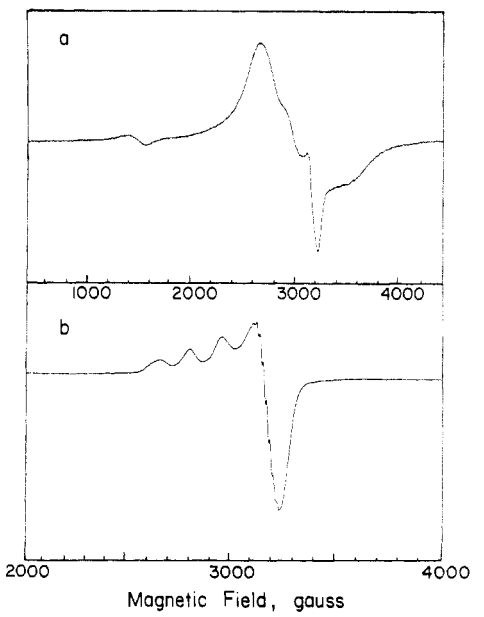

Figure 9. ESR spectra of Cu_2Cu_2SOD (a) in H_2O at pH 5.7 and 140 K, and (b) in 235 mM aqueous KSCN at pH 5.7 and 140 K. Reproduced from ref 44.

NMR spectrum of the nonexchangeable protons in the protein.[23]

Chemical modification studies of SOD with diethyl pyrocarbonate (DEP) independently confirmed the conclusions derived from the NMR results.[55] All eight histidines per subunit in the apoprotein are ethoxyformylated with DEP. Under the same conditions, only a single histidine reacts in native SOD. This residue is presumably histidine-19, which is exposed to the solvent. The NMR spectrum of the modified native enzyme, after reduction of the copper, was identical with that in Figure 8b, indicating that the modified histidine was not one of those observable by NMR spectroscopy. Addition of zinc to apo-SOD led to a decrease in the number of histidines modified by DEP. The average number of modified histidines decreased linearly with increase in the zinc to subunit ratio, reaching one at a ratio of one zinc per subunit. The reactivity of histidines in native SOD and apo-SOD plus one zinc, which reflects their steric accessibility to the reagent, is the same, suggesting similar structures.

NMR studies of the histidine N–H protons in two isozymes of wheat-germ SOD indicated considerable structural homology with the bovine erythrocyte enzyme and suggest a similar role for the zinc.[56] NMR studies of yeast SOD[57] and bovine liver SOD[58] have also been carried out and indicate similarities in the active site structures of SOD from the various sources.

Is Zinc Involved in the Catalytic Mechanism?

Recent work on SOD raises questions about the proposed catalytic mechanism (Figure 2). Addition of thiocyanate ion to Cu_2Cu_2SOD breaks the imidazolate bridge. As shown in Figure 9,[44] the ESR signal of the thiocyanate complex is characteristic of a single type of mononuclear copper, indicating that both copper ions

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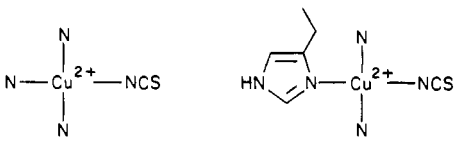
of Cu_2Cu_2SOD must be in very similar environments. The splittings on the $g_\perp$ signal most likely result from nitrogen superhyperfine interactions since the number of lines decreases when $^{15}NCS^-$ is substituted for $^{14}NCS^-$ ion. ESR spectra of the thiocyanate complexes of Cu_2Zn_2SOD and Ag_2Cu_2SOD are identical with that of Cu_2Cu_2SOD, providing independent evidence that, in the presence of thiocyanate, both copper sites in Cu_2-Cu_2SOD are nearly the same. A proposed structure for the thiocyanate complex of Cu_2Cu_2SOD is shown below,



where N stands for an imidazole nitrogen and the imidazole ring depicted is from histidine-61. Both copper ions have the same set of ligands, assuming that the zinc site ligand aspartate has been replaced by thiocyanate, and are likely to be approximately square planar.

Since Cu_2Cu_2SOD is catalytically active in the presence of thiocyanate under conditions where the imidazolate bridge is broken,[44] an intact bridge is not essential to enzymatic activity. The imidazolate group or some other ligand may still be lost from the copper upon reduction to provide a vacant coordination site for inner-sphere reoxidation by superoxide ion, however. Ag_2Cu_2SOD is catalytically inactive with or without thiocyanate present, indicating that the zinc site copper is not responsible for the observed activity. Cu_2E_2SOD is catalytically active under conditions where copper migration (see below) does not occur.[44,59] This result further supports the conclusion that the proposed role of the zinc in modifying the pK_a of the pyrrole hydrogen of histidine-61 is not essential to the mechanism.

Azide ion, unlike thiocyanate, binds to the copper of SOD without displacing the imidazolate bridge.[44,60] Azide could displace water or one of the other protein ligands to the copper. The paramagnetic contribution of Cu(II) to the observed water relaxation rate is greatly reduced in the presence of azide,[61] suggesting simple substitution of the water ligand. An indirect effect, where azide replaces one of the imidazole groups in the equatorial plane followed by loss of the axial water, has also been suggested.[62] The possibility that azide binding to copper in the water position might switch the principal axes, interchanging axial and equatorial sites without displacing a histidine ligand, was not considered, however. Since azide is a competitive inhibitor of SOD,[63] identification of its binding site is important because it is likely to be the site of superoxide ion coordination.

The difference between the binding of azide and thiocyanate to Cu_2Cu_2SOD might arise from different stereochemical interactions between the two copper-coordinated anions and groups in the active site beyond the first coordination sphere of the metal ions. ESR spectra of $[Cu_2(im)\subset A']^{3+}$ in the presence of thiocyanate and azide, however, showed that thiocyanate breaks the bridge under conditions where azide does not.[44] The two anions thus behave the same toward the model compound and the enzyme, indicating that their interaction with the coordination sphere of the imidazolate-bridged binuclear unit, and not other features of the protein's active site, is sufficient to determine their binding mode. These experiments further demonstrate the value of comparative studies of the enzyme and model compounds.

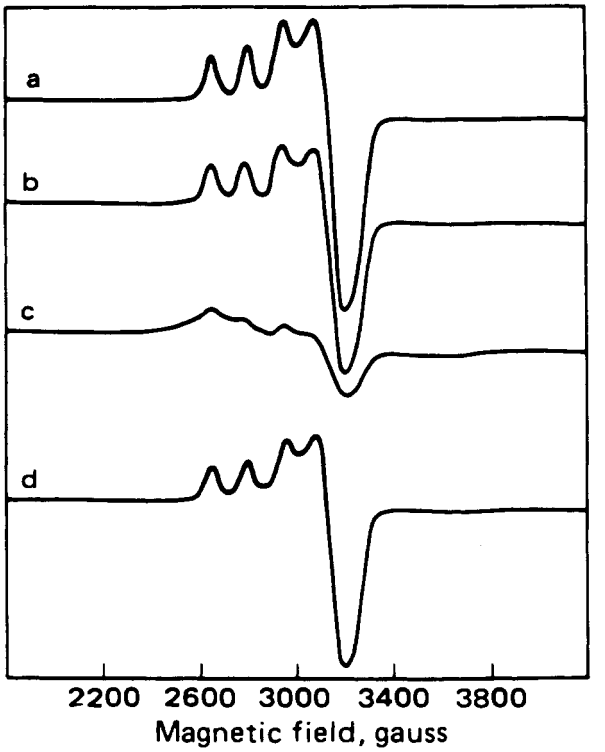


Figure 10. pH dependence of the ESR spectrum of Zn-free SOD at 77 K. The concentration of protein subunits was 2.0 mM in 2.0 ml of 0.1 M potassium phosphate initially buffered at pH 7.8. Curves: a, ESR spectrum of the initial solution; b (pH 8.3) and c (pH 9.3), after addition of 10 and 20 μl of 1 M NaOH; d, after pH was readjusted downward from 9.3 to 6.5 by addition of 34 μl, of 1 M HNO_3. Reproduced with permission from ref 59. Copyright 1979, National Academy of Sciences.

The imidazolate bridge in Cu_2Cu_2SOD is also broken when the enzyme is lyophilized from aqueous solution.[64] Disruption of the bridge, which is reversed when the solid is redissolved, can be prevented by addition of sucrose to the solution prior to lyophilization. As few as 30 molecules of sucrose per molecule of protein are effective. ESR spectroscopy also reveals changes in the copper environment in Cu_2Zn_2SOD on lyophilization that could be the result of breaking the imidazolate bridge.

Copper Migration

Titration of Cu_2E_2SOD to alkaline pH results in a reversible change in the ESR spectrum of the enzyme, as shown in Figure 10.[59] The typical mononuclear copper signal at pH 7.8 is replaced by a broad signal characteristic of an imidazolate-bridged dicopper complex. The spectrum at high pH also has the weak signal

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from the $\Delta M_s = \pm 2$ transition. This result indicates that copper has migrated from the native copper site of one subunit to the vacant zinc site of another subunit, forming the imidazolate-bridged binuclear unit found in Cu_2Cu_2SOD. The disappearance of the Cu_2E_2SOD signal fits a titration curve for a single ionizable group with a pK_a of 8.2. This pK_a could represent the deprotonation of histidine-61, a necessary step in forming the bridged species. The visible absorption spectrum of Cu_2E_2SOD changes at high pH to that characteristic of Cu_2Cu_2SOD, providing additional confirmation of the interpretation of the ESR results.

The migration of copper at pH >7 means that studies of Cu_2E_2SOD conducted at alkaline pH were actually carried out on dicopper subunits and apoprotein subunits. Earlier work on the activity and thermal stability of Cu_2E_2SOD must therefore be reevaluated. The specific activity of Cu_2E_2SOD at pH 6.0, where no dicopper active sites are present, was found to be 80% of that of Cu_2Zn_2SOD, confirming that zinc is not essential to enzymatic activity.

Visible and ESR spectra indicate that, below pH 4, Cu_2Zn_2SOD and Cu_2Cu_2SOD lose the metal in the zinc site giving Cu_2E_2SOD.[43] The different metal binding preferences of the copper and zinc sites at low pH makes possible the preparation of metal-substituted derivatives of SOD having specific metals in each site. The difference in metal binding properties of the two sites and the metal migration observed at pH >7 may be important if the true function of the protein is something other than superoxide dismutation.[65]

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Toward the Future

We have shown the value of model compounds in elucidating the properties of the imidazolate-bridged bimetallic center in SOD. Especially important were the binucleating macrocycles A and A′ which stabilized the $Cu_2(im)^{3+}$ unit over the physiological pH range in aqueous solution. Using ligands of this kind it should be possible to build models for other ligand bridged bimetallic centers in biology. A step in this direction has been taken with the synthesis and characterization of $[Cu_2(OH)(ClO_4)\subset A]^{2+}$. This monohydroxo-bridged strongly antiferromagnetically coupled dicopper(II) complex has spectroscopic and magnetic properties similar to those of binuclear copper sites in other proteins.[66] Two metals in a binucleating macrocycle could also promote new chemistry of significance to areas apart from biological ones. The future of this field holds considerable promise.

The work described here was in large part carried out in the laboratory of S.J.L. with generous support from the National Institute of General Medical Sciences and the National Science Foundation. Several talented graduate students and postdoctoral associates cited in the individual references contributed in a major way to its success. S.J.L. is especially grateful to Professor Joan Valentine for stimulating discussions at the outset and fruitful collaboration in some of the biochemical studies. K.G.S. gratefully acknowledges leave time from Bryn Mawr College to carry out experiments on SOD. We also thank Professors Jean-Marie Lehn, John Bulkowski, and Dr. Andrea Martin for providing generous quantities of the ligands A and A′. Figure 1 was prepared by Matthew Eichner.

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Some Properties of the Phosphorothioate Analogues of Adenosine Triphosphate as Substrates of Enzymic Reactions

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Nucleoside triphosphates, most frequently adenosine triphosphate, play a central role in the bioenergetics of the cell. As shown in Figure 1, the formation of ATP from ADP and inorganic phosphate is coupled with energy-producing reactions such as oxidation through the electron-transport system, photophosphorylation, and glycolysis. Conversely, the expenditure of the chemical potential energy of the nucleoside triphosphates by conversion to diphosphates and inorganic phosphate, or alternatively the monophosphate and inorganic pyrophosphate, is coupled to many energy-requiring processes. Those processes that are characterized by the transfer of chemical energy derived from hydrolysis of nucleoside triphosphate to other forms of energy, such as mechanical work in muscle contraction, translocation during protein synthesis, or ion transport by the Na^+,K^+-ATPase, are of necessity complex processes of cellular organelles. However, those processes in which chemical energy is transferred for endergonic chemical reactions, such as biosynthesis

Mildred Cohn was born in New York, NY, in 1913. She received her undergraduate education (B.A.) from Hunter College and her Ph.D. in physical chemistry from Columbia University. She spent 8 years in the biochemistry department of Cornell Medical College and 14 years in the biochemistry department of Washington University School of Medicine. Since 1960 she has been at the University of Pennsylvania, where for many years she was a Career Investigator of the American Heart Association and currently she is the Benjamin Rush Professor in the department of biochemistry and biophysics. Her interests lie in enzymology, in particular in the mechanism of enzyme action involving adenosine triphosphate as substrate in phosphoryl-transfer reactions of glycolysis, in adenylyl transfer in biosynthetic pathways, and in hydrolytic reactions coupled to energy transduction. Particular emphasis is placed on the use of NMR to study the details of these reactions.

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