(+)-2-desoxystemodinone and for X-ray crystal structure determinations of **3** and **11**, respectively. The X-ray crystal structure of **9** was kindly provided by Dr. C. Campana, Nicolet Analytical Instruments (X-ray Division). Financial support for this research was provided by the National Science Foundation (CHE-8101223), and funds that assisted with the purchase of a Bruker AM-400 NMR spectrometer were obtained from the National Science Foundation (CHE-8216190) and the M. J. Murdock Charitable Trust.

Supplementary Material Available: Experimental data on compounds **3** and **7-18** (5 pages). Ordering information is given on any current masthead page.

Preparation and Characterization of Cu_2Ni_2 and Ag_2Ni_2 Superoxide Dismutase, Two New Metal-Substituted Derivatives

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Bovine copper-zinc superoxide dismutase, Cu_2Zn_2SOD, is a metalloprotein of molecular weight 31 200 which is comprised of two equivalent subunits, each of which contains a Cu^{2+} and a Zn^{2+} ion bound in close proximity and bridged by the imidazolate ring of a histidyl residue.[1] Spectroscopic studies of derivatives with Co^{2+} substituted at the zinc site have provided considerable information concerning the nature of this site.[1] We describe here the preparation and characterization of two new derivatives in which Ni^{2+} has been substituted at this same site. While the geometric preferences and ionic radii of Zn^{2+}, Co^{2+}, and Ni^{2+} are quite similar, their magnetic and spectroscopic properties are very different, and comparisons of results obtained from studies of each of these metal ions in identical or nearly identical ligand environments, including metailoprotein metal-binding sites,[3] have frequently provided complementary information, particularly in NMR studies.[2,3] The first new derivative is Cu_2Ni_2SOD, which contains Cu^{2+} in the native copper site and Ni^{2+} in the native zinc site. This derivative is EPR silent (at 90 K) and has a particularly rich ^1H NMR spectrum (at ambient temperature) consisting of isotropically shifted resonances from ligands bound to both metal ions. These observations are due to the fact that the fast relaxing electrons of the paramagnetic Ni^{2+} ion interact with the unpaired electron on the Cu^{2+} ion, causing its relaxation rate to increase. A similar phenomeon has been reported by Bertini and co-workers[4] for Cu_2Co_2SOD. The second new derivative is Ag_2Ni_2SOD, which contains Ag^+ in the native copper site and Ni^{2+} in the native zinc site and has allowed us to determine the spectroscopic properties of Ni^{2+} when bound to that site.

Cu_2E_2SOD and Ag_2E_2SOD, derivatives in which either Cu^{2+} or Ag^+ is bound to the native copper site and the zinc site is empty (E = empty), were prepared in acetate buffer as previously reported.[5] The buffer was then changed to 50 mM phosphate, pH 6.5, and 2 equiv of Ni^{2+} was infused directly into each solution. For either solution, an increase of the absorbance near 500 nm indicated the formation of Ni-substituted SOD as shown in Figure 1A,E. The visible λ_{max} at ~700 nm in Cu_2E_2SOD was essentially unchanged by the addition of Ni^{2+}, indicating that Cu^{2+} remains

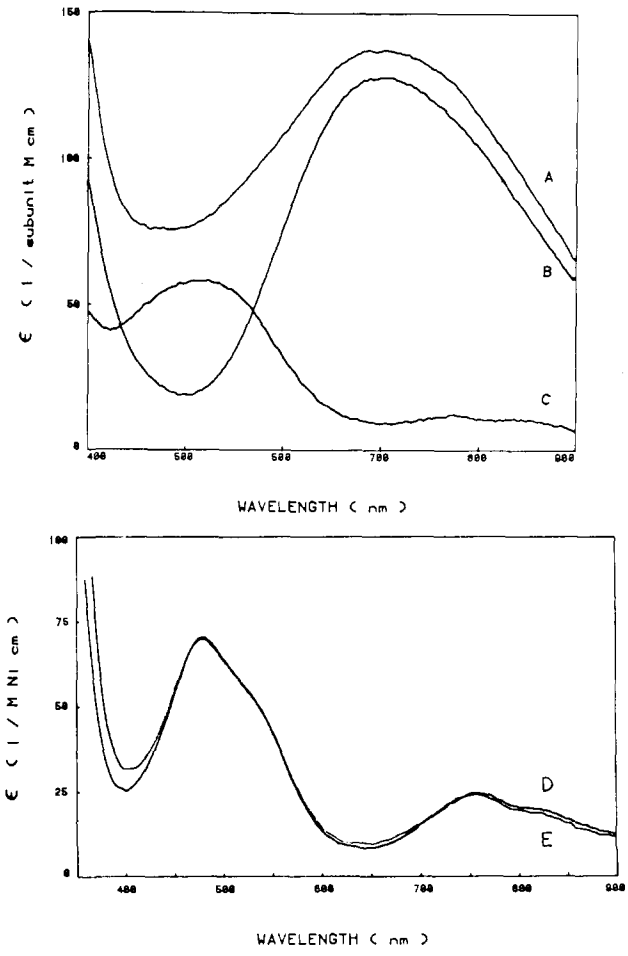

Figure 1. Electronic absorption spectra at room temperature of (A) Cu_2Ni_2SOD and (B) Cu_2E_2SOD, (C) the difference of Cu_2Ni_2SOD and Cu_2E_2SOD, (D) reduced Cu_2Ni_2SOD, and (E) Ag_2Ni_2SOD. Solutions were in 50 mM phosphate buffer, pH 6.5, referenced against deionized water.

in the copper site rather than migrating to the zinc site where it would be expected to give a λ_{max} instead at ~800 nm.[6] This conclusion is also supported by the observation that addition of azide or cyanide ion produced new absorption maxima at 475 and 530 nm, respectively, characteristic of derivatives in which Cu^{2+} is bound at the native copper site when those anions are bound to the protein.[1,4] The virtual disappearance (<5% remaining) of the EPR signal of Cu_2E_2SOD upon addition of 2 equiv of Ni^{2+} indicates that almost all of the Ni^{2+} was bound in the zinc site under those conditions. Addition of 2 equiv of Zn^{2+} to solutions of either oxidized or reduced Cu_2Ni_2SOD resulted in the complete disappearance of the absorbance near 500 nm, indicating that Zn^{2+} had displaced Ni^{2+} from the zinc site.[7] The Cu_2NiSOD derivative was found to have 26-45% of the activity of native SOD at pH 7.8[8] by using the cytochrome C-xanthine assay.[9]

The paramagnetically shifted ^1H NMR spectrum of Cu_2Ni_2-SOD obtained in H_2O by the modified DEFT pulse sequence[10] on a Brucker WP200 spectrometer is shown in Figure 2B. At least 20 signals are detected (occurring over a range of about 120

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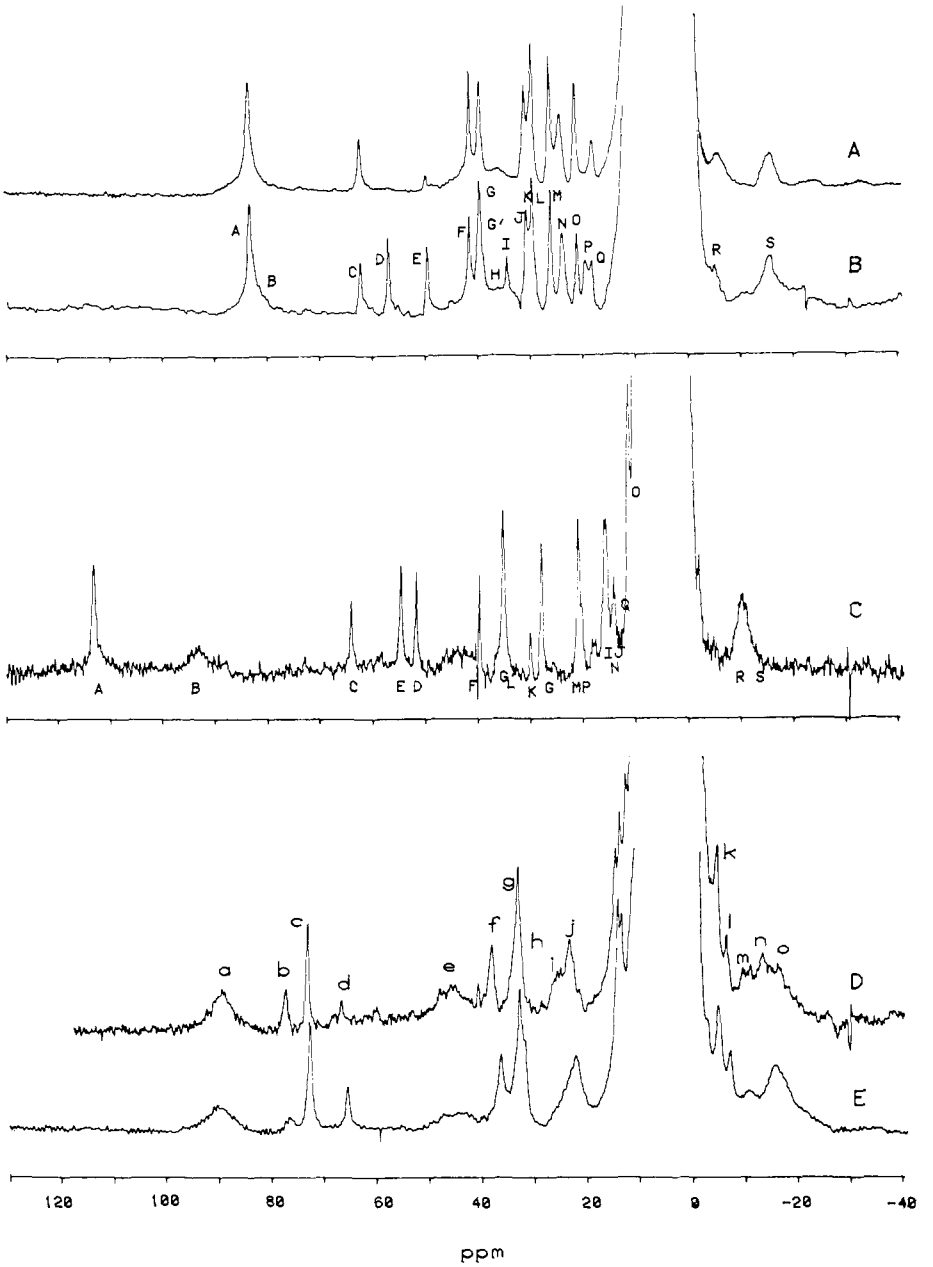

Figure 2. ^{1}H NMR spectra (200 MHz) at 298 K of (A) Cu_2Ni_2SOD in D_2O, (B) Cu_2Ni_2SOD in H_2O, (C) Cu_2Ni_2SOD in H_2O with saturating amounts of sodium azide added, (D) reduced Cu_2Ni_2SOD in H_2O, and (E) Ag_2Ni_2SOD in H_2O. Solutions were in 50 mM phosphate buffer, pH or pH* 6.5.

ppm) which are due to the amino acid residues coordinated to both the Cu^{2+} and the Ni^{2+} ions. The long electronic relaxation time of the d^9 metal ion Cu^{2+} in Cu_2Zn_2SOD causes the ^{1}H NMR signals of that portion of the protein near to the copper-binding region to be broadened beyond detection.[3] However, the proximity of the two metal ion binding sites in each subunit, bridged by an imidazolate ring from a histidyl residue, provides a means of altering the relaxation properties of the Cu^{2+} ion[11] as has previously been observed by Bertini et al.[4] for Cu_2Co_2SOD. The ligands that bind the two metal ions in each subunit of the native protein consist of five histidyl imidazoles, one histidyl imidazolate that acts as a bridge between the two metal ions, and one aspartyl carboxylate. Resonances due both to imidazole C–H and N–H protons may be observed by NMR and may be distinguished by comparison of their ^{1}H NMR spectra in H_2O and D_2O since the N–H protons are readily exchangeable if they are accessible to solvent (signals D, E, I, and P in Figure 2B). A decrease of signal G occurs when the derivative is in D_2O for a longer time or is prepared from D_2O buffer, indicating the presence of another slowly solvent-exchangeable signal, G′, in Figure 2B. Addition of increasing amounts of azide ion to Cu_2Ni_2SOD (see Figure 2C) causes certain of the isotopically shifted resonances (A, B, D, E, G, I, J, L, M, N, O, and Q) to shift. Assuming that azide binds only to the copper ion in this derivative, as it does in the other characterized derivatives in which Cu^{2+} is bound to the native copper site,[1,4] we conclude that the azide ion is perturbing only those protons on ligands bound to copper and not on those bound to nickel. We therefore tentatively assign these resonances to histidyl residues at the native copper site. It is particularly interesting to note that signals I, J, O, and Q move to the diamagnetic region of the spectrum in the presence of azide, suggesting that a ligand–metal bond may have been broken. This observation strongly resembles that observed for azide binding to Cu_2Co_2SOD and has been interpreted as being due to dissociation of a histidyl imidazole ligand, possibly His-44, from the Cu^{2+} center upon azide binding.[4]

The visible electronic absorption spectra of reduced Cu_2Ni_2SOD and of Ag_2Ni_2SOD can be seen in Figure 1D,E to be extremely similar to each other and to the difference spectrum of Cu_2Ni_2SOD minus Cu_2E_2SOD (Figure 1C). The relatively long wavelength

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d-d bands due to the Ni^{2+} chromophore are consistent with a tetrahedral or distorted tetrahedral geometry,[12] as is generally observed for metal ions bound to the native zinc site of this protein.[1] Addition of azide to either of these derivatives causes no change in their spectra, consistent with the absence of a ligand-binding site on the Ni^{2+} ion when it is bound to the native zinc site. This behavior also is similar to that observed when other metal ions are bound to that site.

The ^1H NMR spectra of Ag_2Ni_2SOD (Figure 2E) and of reduced Cu_2Ni_2SOD (Figure 2D) are also extremely similar. There are 15 detectable paramagnetically shifted proton NMR signals which we assign to the three histidyl and one aspartyl residues, which are presumably coordinated to Ni^{2+}. By considering the line width (which is contributed by the distance-dependent hyperfine interactions and chemical exchanges) of the isotropic shifts, the sharper signals can be tentatively assigned to C_δ-H or N-H protons and the broad signals to C_ϵ-H protons.[3] We assign resonances, b, h, and j to N-H protons since they are not observed in D_2O. The upfield-shifted signals are tentatively assigned as C_β-H_2 protons of coordinated histidyl and aspartyl residues by comparison with paramagnetically shifted resonances of other proteins.[3] Several other Ni^{2+}-substituted derivatives of SOD have also been prepared in our laboratory. Their characterization and a fuller assignment of the NMR spectra of the above-described nickel derivatives are now in progress.

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21-Thiatetra-*p*-tolylporphyrin and Its Copper(II) Bicarbonate Complex. Structural Effects of Copper-Thiophene Binding

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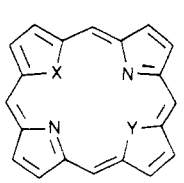
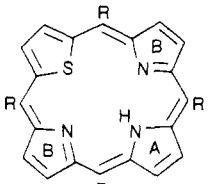
Replacement of the nitrogen atoms of porphyrins with other potential donors produces macrocycles with differing central cavity sizes whose complexing abilities remain unknown. Limited data regarding the oxa- and thiaporphyrins **1** and **2** are available,[1] while the dithia- and diselenaporphyrins **3** and **4** have received somewhat more attention.[2] Here we report the preparation and structural characterization of 21-thiatetra-*p*-tolylporphyrin (**5**, STTPH) and its copper(II) complex. This represents the first thorough structural characterization of a heteroporphyrin system. The structure of the copper complex is of particular interest because of the limited coordinating ability of the thiophene moiety,[3] the geometric constraints imposed by the macrocycle, and the multiplicity of possibilities (S-bound, C-bound, η^5-bound) available for metal/thiophene coordination.[4]



1, X=O, Y=NH
2, X=S, Y=NH
3, X=Y=S
4, X=Y=Se



5, R = *p*-$CH_3C_6H_4$

The condensation of 2,5-bis(*p*-tolylhydroxymethyl)thiophene[5] with 2 mol of *p*-tolylaldehyde and 3 mol of pyrrole in boiling propionic acid conducted over 1 h, followed by cooling, produces a mixture from which STTPH, $TTPH_2$ (tetra-*p*-tolylporphyrin), and traces of S_2TTP (**3**) crystallize on standing for 24 h. The solid material was dissolved in carbon tetrachloride and subjected to chromatography on basic alumina. Two major bands eluted to give $TTPH_2$ and STTPH successively. The faction containing STTPH was evaporated, and the product recrystallized from dichloromethane/ethanol to give STTPH in 5% yield (electronic spectrum which is porphyrin-like: UV-vis λ_{max} 428 (Soret), 514, 550, 618, 680 nm; ^1H NMR spectrum (360 MHz, $CDCl_3$, 25 °C) δ 9.785 (s, thiophene), 8.924 (d, J = 2.16 Hz, pyrrole A), 8.673, 8.599 (q, J = 4.5 Hz, pyrrole B), 8.129, 8.066 (s, ortho phenyl), 7.612, 7.535 (meta phenyl), 2.697 (s, *p*-methyl), -2.661 (t, J = 2.16 Hz, NH)). The NMR spectrum unambiguously locates the N-H group on pyrrole ring A as indicated by the long-range coupling to the adjacent C-H groups and the characteristic downfield shift.

The structure of STTPH was investigated through an X-ray crystal structure.[6] The molecule is planar, as can be seen in Figure 1. Because of the presence of sulfur, the core size of the macrocycle is restricted: the nonbonded S(1)···N(1′) distance is 3.585 Å while the N(2)···N(2′) distance is 4.383 Å (somewhat longer than the tetragonal form (4.108 Å)[7a] or the triclinic form (4.06, 4.20 Å)[7b] of tetraphenylporphyrin).

Insertion of copper(II) was achieved by boiling a mixture of STTPH in chloroform and an ethanolic solution of copper(II) chloride hydrate in the presence of triethyl orthoformate and solid sodium carbonate for 0.5 h. After evaporation of the solution, the residue was subjected to chromatography on neutral alumina with dichloromethane as eluent, and the product, Cu(STTP)(C_3H) (**6**; yield 70%), was recrystallized from dichloromethane/toluene (λ_{max} 466 (Soret), 580, 630, 710 nm).

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(6) Dark-blue parallelepipeds of STTPH (**5**) were grown by diffusion of ethyl ether into a dichloromethane solution of the macrocycle. They belong to the monoclinic space group $P2_1/n$ (No. 14) with a = 9.484 (2) Å, b = 9.305 (2) Å, c = 21.370 (5) Å, β = 100.04 (2)°, and Z = 2 at 130 K. Refinement of 1699 reflections with $I > 2\sigma I$ and 249 parameters gave R = 0.051. There is a center of symmetry at the center of the macrocycle; hence the sulfur atom is disordered. The major form involving S(1) with 0.38 occupancy is shown in Figure 1; its centrosymmetrically related form interchanges the approximate positions of S(1) and N(1′). The minor form with 0.12 occupancy for each of the centrosymmetrically related positions places the sulfur atom near N(2) (or N(2′)).

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