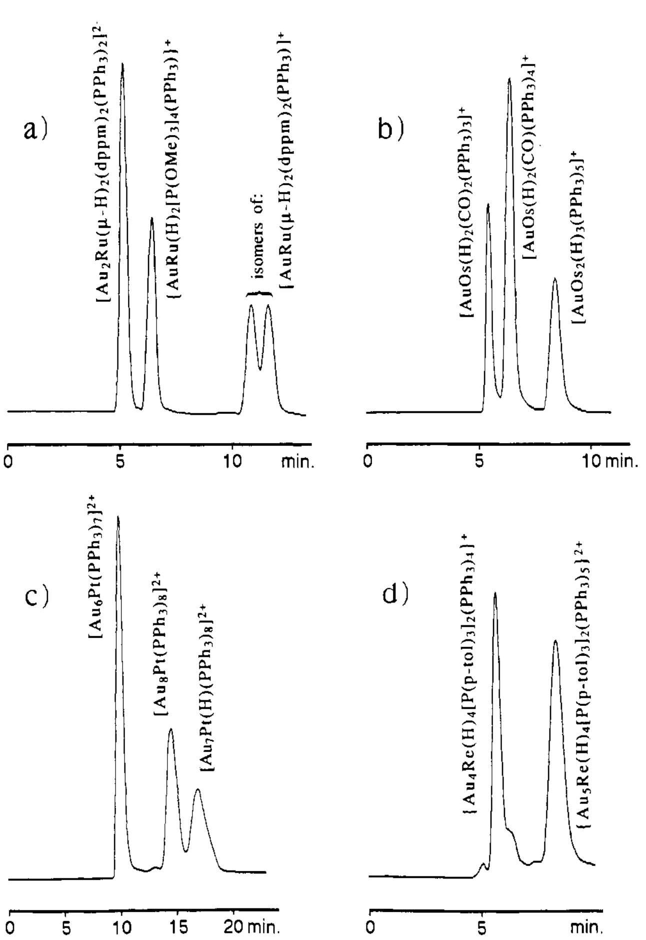

Figure 2. Chromatograms of mixed-metal–gold clusters run at a flow rate of 0.5 mL/min with the following solvent conditions: (a) linear gradient changing from 20% 0.125 M NH_4PF_6, 65% EtOH, and 15% CH_3CN to 5% 0.125 M NH_4PF_6, 75% EtOH, and 20% CH_3CN in 15 min; (b) 10% 0.125 M NH_4PF_6, 70% EtOH, and 20% CH_3CN; (c) 20% 0.125 M NH_4PF_6, 60% EtOH, and 20% CH_3CN; (d) 15% 0.125 M NH_4PF_6, 60% EtOH, and 25% CH_3CN.

is often much easier to grow crystals of a cluster after purification by preparative-scale HPLC.

Conclusion

Reversed-phase HPLC chromatography on silica gel C18 has proven to be a useful technique for the separation and identification of cationic gold and a variety of mixed-metal-gold cluster compounds. Only minor changes in solvent composition have been necessary for the successful separation of these different mixed-metal clusters. HPLC has also been shown to be a fast and very sensitive technique for the detection of reactions and identification of compounds, and preparative HPLC is a convenient way of separating different reaction products.

Acknowledgment. This work has been supported by the Netherlands Foundation for Chemical Research (SON), the National Science Foundation (Grant CHE 851923), and the donors of the Petroleum Research Fund, administered by the American Chemical Society. S.Y. acknowledges partial support from the University of Minnesota China Center.

Registry No. $[Au_9(PPh_3)_8]^{3+}$, 60477-23-8; $[Au_6(PPh_3)_6]^{2+}$, 60477-20-5; $[Au_8(PPh_3)_7]^{2+}$, 74245-04-8; $[Au_8(PPh_3)_8]^{2+}$, 72187-44-1; $[Au_{11}(PPh_3)_8(CNO)_2]^+$, 98921-32-5; $[AuRu(\mu\text{-}H)_2(dppm)_2(PPh_3)]^+$, 108969-21-7; $[Au_2Ru(\mu\text{-}H)_2(dppm)_2(PPh_3)_2]^{2+}$, 102538-83-0; $\{AuRu(H)_2[P(OMe)_3]_4(PPh_3)\}^+$, 108969-25-1; $[AuOs(H)_2(CO)_2(PPh_3)_3]^+$, 112681-80-8; $[AuOs(H)_2(CO)(PPh_3)_4]^+$, 112681-81-9; $[AuOs_2(H)_3(PPh_3)_5]^+$, 112681-82-0; $[Au_6Pt(PPh_3)_7]^{2+}$, 107712-38-9; $[Au_7Pt(H)(PPh_3)_8]^{2+}$, 110870-02-5; $[Au_8Pt(PPh_3)_8]^{2+}$, 110870-04-7; $\{Au_4Re(H)_4[P(p\text{-}tol)_3]_2(PPh_3)_4\}$, 107712-40-3; $\{Au_5Re(H)_4[P(p\text{-}tol)_3]_2(PPh_3)_5\}$, 107742-33-6.

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Electronic Relaxation of a Copper(II) Dimer in a Macromolecular Complex As Evaluated from Solvent Proton Relaxation

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Received March 16, 1987

Bovine erythrocyte superoxide dismutase (SOD) is a dimeric enzyme containing one copper and one zinc per monomeric subunit.[2] The structure of the copper–zinc region, as found by X-ray crystallography, is shown in Figure 1. The protein can be readily demetalated, and a variety of metal-substituted forms have been described.[3] The Cu^{2+} ions within each monomer of dimeric Cu_2Cu_2SOD are antiferromagnetically coupled, with $2J = -52$ cm^{-1} and an $S = 0$ ground state. However, they have an EPR spectrum typical of an $S = 1$ system,[4] arising from population of the excited states. We have now investigated the apoSOD/Cu^{2+} system by measuring the longitudinal magnetic relaxation rate $1/T_1$ of water protons in solutions containing apoSOD plus increasing amounts of Cu^{2+}, at field values ranging from 0.23 mT to 1.17 T (0.01–50 MHz proton Larmor frequency), and at 25 °C, which corresponds to $kT \gg J$. The variation of $1/T_1$ with field is known as the nuclear magnetic relaxation dispersion (NMRD) profile.[5-7] The theory is now available to interpret NMRD profiles of Cu^{2+} systems[6,7] by using as adjustable parameters (1) r_{Cu-H}, the Cu^{2+}–proton separation of the inner-coordinated waters, (2) θ, the average angle of the metal–proton vector with respect to the z axis of the electron–copper nucleus hyperfine coupling tensor (assumed to be axial), and (3) the correlation time of the Cu^{2+}–proton magnetic dipolar interaction (assumed to be field independent), which is dominated by the electronic relaxation time τ_S in Cu^{2+}–protein systems.[6] The present aim is to measure the correlation time of magnetically coupled Cu^{2+} systems and—by equating it to τ_S—evaluate the extension of the now well-established theory to the more complex case of magnetically coupled systems.[6,8] These points are key for understanding the electronic structure of coupled dimers of paramagnetic ions, in a general sense. In addition, NMRD profiles are very sensitive to the uptake of copper ions by apoSOD, allowing one to obtain protein-specific biochemical information on the sequential binding of Cu^{2+} to the copper and zinc sites of the native enzyme and on the respective binding affinities.

Materials and Methods

Native SOD was purchased from Diagnostic Data Inc., Mountain View, CA. Apoprotein was prepared as previously reported;[10,11] recon-

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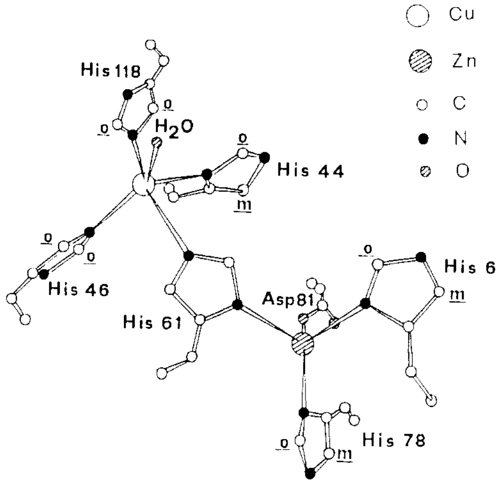

Figure 1. Configuration of the ligands of the metal ions of native Cu_2Zn_2SOD.

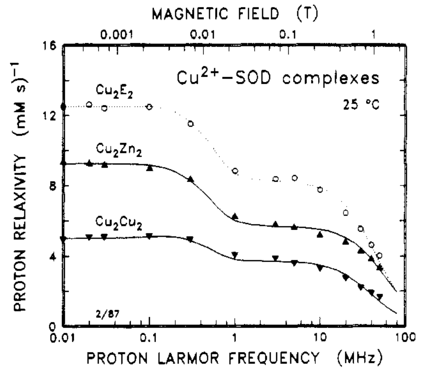

Figure 2. Paramagnetic contribution to the $1/T_1$ NMRD profiles of solutions of three complexes of Cu^{2+} ions with bovine superoxide dismutase, at 25 °C. The data are expressed as relaxivity, the increment in the rate per millimolar increase of dimeric protein. The major result is the relative amplitude of the profiles for the two similarly liganded complexes, Cu_2Zn_2SOD and Cu_2Cu_2SOD: replacing Zn^{2+} at the zinc site by Cu^{2+} reduces the relaxivity by half. The result is expected from theory,[8,18,19] assuming no direct relaxivity contribution from Cu^{2+} at the zinc sites, i.e., no exchanging waters. The profile for Cu_2E_2SOD, a complex with unoccupied zinc sites, has a higher relaxivity, attributable to a shortened Cu^{2+}–proton separation.[16]

stituted Cu_2Zn_2SOD was prepared by addition of copper at pH 3.8 followed by addition of zinc at pH 5.3.[12] Aliquots of 20 mM Cu^{2+} were added to 0.5 mL of a 0.44 mM solution of (dimeric) apoSOD at pH 5.3 to make Cu_2Cu_2SOD. The NMRD measurements were obtained on a field-cycling relaxometer, as previously described.[13]

Results

The NMRD profiles for Cu_2E_2SOD (E for "empty"), reconstituted Cu_2Zn_2SOD, and Cu_2Cu_2SOD are shown in Figure 2. The profile of the reconstituted SOD is comparable to, but somewhat greater in magnitude than, that previously reported for somewhat different experimental conditions of buffer and pH.[14] The data for Cu_2E_2SOD and Cu_2Zn_2SOD have been analyzed by using the theoretical treatment[6] developed for the dipolar interaction between a proton and an unpaired electron when the electronic magnetic levels are further split by a hyperfine interaction with the nucleus of the paramagnetic metal ion. For the general case that is of interest here, i.e., for $A_\parallel > A_\perp > 0$, only numerical solutions of the relaxation equations are available.[6] Therefore, the present data were related to theory by choosing approximate initial values for r_{Cu-H}, τ_S, and the mean angle θ and then calculating the predicted NMRD profile and comparing it to the data, readjusting the initial values, and iterating this procedure until a best fit of data and theory were obtained. The results for Cu_2Zn_2SOD (cf. Table I) are that r_{Cu-H} is 3.44 Å and that $\tau_S = 2.8 \times 10^{-9}$ s. The fitting assumes one exchanging water; $A_\parallel = 143 \times 10^{-4}$ cm^{-1} and $A_\perp = 20 \times 10^{-4}$ cm^{-1} for the parallel and perpendicular components of the electronic–nuclear hyperfine interaction of the Cu^{2+} ions. The latter value differs somewhat from that found from single-crystal EPR data,[15] which were obtained under different experimental conditions. Using the published value of $A_\perp$ (43×10^{-4} cm^{-1}) gives a somewhat poorer fit, although the change in r_{Cu-H} is only about 3%. Similarly, since the measured rates depend on the inverse sixth power of r_{Cu-H}, its derived value is rather insensitive to $1/T_1$; a 20% uncertainty in the scale of $1/T_1$ contributes only 3% uncertainty to r_{Cu-H}. The value obtained for r_{Cu-H}, as previously noted,[6] is consistent with a semicoordinated water molecule, whereas the derived value for τ_S is typical of non-blue copper proteins.

Table I. Results of Comparisons of Relaxation Theory with the NMRD Profiles of Various Cu^{2+}–Superoxide Dismutase Complexes, Including One with No Metal Ions at the Zinc Sites

system	r_{Cu-H},[a] Å	τ_S,[b] ns	θ,[c] deg
Cu_2Zn_2SOD[d]	3.44	2.8	19
Cu_2Cu_2SOD[e]	3.37	4.2	29
Cu_2E_2SOD[f]	3.18	3.6	24

[a] Calculated by assuming a single water molecule interacting with each Cu^{2+} in the copper sites, with its protons equidistant from the ion. [b] It is assumed, as in other Cu^{2+}–protein systems,[6,8,21] that τ_S can be equated to the correlation time. [c] θ is the angle between the direction of r_{Cu-H} and the unique axis of the hyperfine tensor for the interaction of a Cu^{2+} ion with its nucleus. This parameter is required by the theoretical treatment,[6,8] but the results are relatively insensitive to its value. [d] Sample unbuffered at pH 5.1; fitting done by using $A_\parallel = 143 \times 10^{-4}$ cm^{-1} and $A_\perp = 20 \times 10^{-4}$ cm^{-1}. [e] Sample unbuffered at pH 5.3, fitting done by using $A_\parallel = 143 \times 10^{-4}$ cm^{-1} and $A_\perp = 20 \times 10^{-4}$ cm^{-1}. [f] Sample unbuffered at pH 5.3, fitting done by using $A_\parallel = 148 \times 10^{-4}$ cm^{-1} and $A_\perp = 20 \times 10^{-4}$ cm^{-1}.

Analysis of the results for Cu_2E_2SOD, with an altered ligand configuration because of the empty zinc site, gives $r_{Cu-H} = 3.18$ Å and $\tau_S = 3.6 \times 10^{-9}$ s (cf. Table I). The derived proton–copper distance is slightly shorter than that for Cu_2Zn_2SOD, as previously proposed,[16] consistent with a more tetragonal ligand environment of the Cu^{2+} ions, as also inferred from EPR data, which indicate a less rhombic chromophore.[17] Even a qualitative inspection of the data indicates that the larger relaxivity of Cu_2E_2SOD compared to that of Cu_2Zn_2SOD must arise from a shorter r_{Cu-H} since τ_S, which determines the inflection of the NMRD profile at high fields, is equal or only slightly larger for Cu_2E_2SOD than for the reconstituted protein.

It is seen in Figure 2 that the relaxivity values of Cu_2Cu_2SOD solutions are about half those of Cu_2Zn_2SOD at all fields. This effect arises from (antiferromagnetic) magnetic exchange coupling of the two close Cu^{2+} ions in each SOD monomer. The effect is very general in such coupled systems, and the exact numerical factor relates to the value of the spin S of the paramagnetic ions; for $S = 1/2$, as in Cu^{2+} ions, the factor is $1/2$. A theory was given quite early for hyperfine interactions by Slichter[18] and has been extended recently by Bertini and collaborators[6,8,19] to nuclear–

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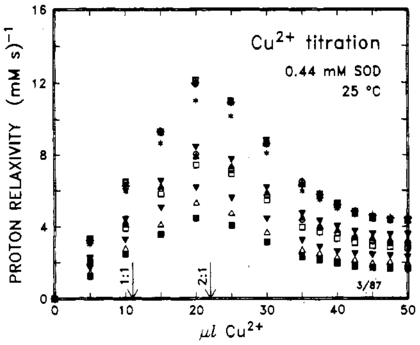

Figure 3. Titration of 20 mM Cu^{2+} into (dimeric) apoSOD. Data at all the fields of Figure 2 are shown, from 0.01 MHz at the top to 50 MHz at the bottom. A clear break in the rates is seen when all the copper sites are filled, indicating strong binding at the Cu^{2+} site; the subsequent monotonic, but nonlinear, decrease indicates weaker binding of Cu^{2+} to the zinc sites as well as the nature of the Cu^{2+}–Cu^{2+} interaction (see text).

electronic dipolar interactions. The underlying physical principle is rather straightforward and can be described rather directly for coupled $S = {}^1/_2$ systems: In the absence of an exchange interaction between the Cu^{2+} ions, the quantization of both the nuclei and the electrons is, to first order, along the direction of the applied field. Thus, the nuclear and electronic moments are always either parallel or antiparallel to each other, and the energy levels and transitions are determined accordingly. When two Cu^{2+} spins become tightly coupled, the quantization of each Cu^{2+} spin is along the exchange field of the other and only the net spin (here, S = 1) is quantized along the external field. The electron spin of a single Cu^{2+} ion is then no longer a good quantum number, and one must look at its orientation in space for each state of the S = 0 and S = 1 manifolds to obtain the nuclear–electronic interaction. Thus, two Cu^{2+} spins combine to give a triplet with S = 1 and total M = 1, 0, −1 and a singlet with S = 0 and M = 0. For the M = 0 levels, i.e., two of the four levels, there is no component of electronic moment along the field direction (along which the nuclei are quantized) and, therefore, no nuclear interaction (either hyperfine[18] or dipolar[8]). Thus, only half the levels give a nonzero interaction with the nuclei for coupled ions with S = 1, and the source of the factor ${}^1/_2$ is immediate. For coupled ions with $S > {}^1/_2$, the appropriate factor is not so obvious and one must resort to the formalism of Bertini et al.,[8] who have tabulated the factor for a range of spin values, assuming that all levels are uniformly populated. Moreover, their formalism can readily be extended to the case when the spacings of the manifolds are comparable to kT.

Thus, it follows that for Cu$_2$Cu$_2$SOD, assuming that its ligand configuration is the same as that of Cu$_2$Zn$_2$SOD, theory predicts that the amplitude of the NMRD profile should be precisely half that of Cu$_2$Zn$_2$SOD when the exchangeable protons on one Cu^{2+} ion are too far from the other Cu^{2+} ion to be affected by its dipolar field. Since the theoretical treatment of the dipolar relaxation of a liganded proton also requires knowledge of the hyperfine coupling between the Cu^{2+} ion and its nucleus, we have used the value of the EPR hyperfine coupling found for the monomer since the decrease in hyperfine interaction due to pairing is incorporated implicitly in the above considerations. For Cu$_2$Cu$_2$SOD, we find the same $r_{\text{Cu-H}}$ as for Cu$_2$Zn$_2$SOD and a slightly longer $\tau_S = 4.2 \times 10^{-9}$ s (cf. Table I). It appears, therefore, that the electronic relaxation time of the Cu^{2+} ion that binds water is not altered detectably when Cu^{2+} is brought into interaction with a nearby, second, Cu^{2+} ion. This important fact has not been uncovered by EPR spectroscopy.

Considering the normalized relaxation rates at selected fields as a function of copper content (Figure 3), we note that the relaxivities increase linearly up to 2:1 copper to dimeric protein ratio (giving no indication of a protein monomer–monomer interaction), then decrease linearly until about 3.5:1, and level off near a ratio of 4:1. The initial decrease is due to the abrupt onset of magnetically coupled pairs of adjacent Cu^{2+} ions once the copper sites are filled and Cu^{2+} ions begin to occupy the zinc sites, combined with the absence of any contribution to the relaxivity from the Cu^{2+} ions in the zinc sites. The titration shows clearly that binding of Cu^{2+} to the native copper sites is quantitative under the present experimental conditions, indicating very tight binding, whereas the subsequent binding to the zinc sites is weaker. An upper limit of 10^{-6} M for the affinity constant of Cu^{2+} for Cu$_2$E$_2$SOD can be estimated from the present data, consistent with previous findings under different conditions.[20,21]

Concluding Remarks

The apoSOD/Cu^{2+} system is particularly tractable for generating magnetically coupled Cu^{2+} pairs in a macromolecular environment, so as to allow refinement of the theoretical basis of electronic relaxation of coupled paramagnetic ions. It has the advantage that the first equivalent of Cu^{2+} binds to the copper site of SOD quantitatively, yielding a single species that can be interpreted unambiguously. In addition, Cu^{2+} enters the zinc site; no direct effect on the NMRD profile is expected since there is no inner-coordinated water there. Rather, the influence of the second Cu^{2+} is through alteration of the interaction between the Cu^{2+} ions in the copper site and the protons they relax. The theory corroborates that this is the dominant effect; in essence, the J splitting of the levels of the paired Cu^{2+} ions (large compared to the widths of the electronic levels, but small compared to kT), alters the quantization direction of the individual Cu^{2+} ions and makes half the levels ineffective in proton dipolar[8] and nuclear hyperfine[18] interactions, causing the relaxivity to decrease by half. By contrast, the electronic relaxation times are not significantly altered in the Cu^{2+} dimer, despite the strong magnetic coupling; i.e., the mechanism that couples the magnetic energy of the paramagnetic ions to the protein is essentially unaltered by the pairing of the two Cu^{2+} ions and the concomitant rapid exchange of electronic spin within the Cu^{2+} dimer. This is a new result and is particularly relevant for understanding NMR, NMRD, EPR, and ENDOR experiments on this and related systems of paired, magnetically coupled, paramagnetic ions.

Registry No. Cu^{2+}, 15158-11-9.

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Semiquinone–Quinone Redox Species Involving Bis(bipyridine)ruthenium(II): Resonance Raman Spectra

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Received July 16, 1987

Haga, Dodsworth, and Lever[2] recently reported the UV/visible, IR, and ESR spectra together with the electrochemical data of a series of complexes belonging to the redox chain RuN$_4$(cat), [RuN$_4$(sq)]$^+$, and [RuN$_4$(q)]$^{2+}$ where the N$_4$ units were bis(bipyridine) or tetrakis(pyridine) and the dioxolene ligands were catechols (cat) and their one- and two-electron oxidation products, semiquinones (sq) and quinones (q), respectively. All data confirmed that these complexes contain ruthenium(II). The electronic spectra were reported and assigned by correlation with the elec-

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