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^{1}H-NMR and relaxometry of copper-containing dimers in proteins

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Summary. The water-proton nuclear-magnetic-relaxation dispersion profiles have been analyzed for Cu_2Zn_2–superoxide dismutase (SOD) and Cu_2–alkaline phosphatase (AP). The electronic relaxation times are derived, together with structural information. The effect of magnetic coupling with another copper ion in Cu_2Cu_2SOD and Cu_2Cu_2AP is discussed. It is shown that the electronic relaxation times of copper(II) essentially do not change. The opposite happens with Cu_2Co_2SOD, Cu_2Co_2AP and Cu_2Ni_2SOD in which fast-relaxing metal ions provide relaxation mechanisms for copper(II) as well. In these cases the systems can be studied through high-resolution NMR spectroscopy.

Key words: Copper – Cobalt – Nickel – Superoxide dismutase – Alkaline phosphatase – NMR – Relaxometry – Nuclear relaxation – Electronic relaxation

Introduction

Type II copper(II) in proteins is often solvent-exposed and suitable for water ^{1}H T_1^{-1} NMR measurements at various magnetic fields (nuclear magnetic relaxation dispersion, NMRD, or relaxometry; Koenig and Brown III 1987). We are interested here in discussing the electronic relaxation properties of copper(II) as they are affected by magnetic interactions with other metal ions. Sometimes the electronic relaxation properties of copper(II) are so largely affected that high-resolution NMR spectra can be measured. The protein matrices that we have used for analyzing the effect of magnetic coupling are copper-zinc superoxide dismutase (SOD; Gärtner and Weser 1986) and alkaline phosphatase (AP; Coleman and Gettins 1983). The former protein is a dimer containing a copper and a zinc ion in each subunit; the metal site is shown in Fig. 1 (Tainer et al. 1982). The latter protein is a dimer containing two zinc ions and one magnesium ion in each subunit. The zinc sites of pertinence here, called A and B, are depicted in Fig. 2 (Wyckoff 1983). Magnesium is bound to site C. The systems investigated are Cu_2Zn_2, Cu_2Cu_2, Cu_2Co_2 and Cu_2Ni_2SOD, and Cu_2E_2 (E = empty), Cu_2Cu_2 and Cu_2Co_2AP (the first metal in the name always refers to the native copper site in SOD, and to the A site in AP). The water ^{1}H-NMRD measurements provide T_1^{-1} values of solvent protons as a function of the external magnetic field, B. The latter can vary with our machine between 0.01–50 MHz (Koenig and Brown III 1987). The experimental data are subtracted for the diamagnetic value, i.e. the value of solutions of protein without copper, in such a way as to obtain the paramagnetic contribution, T_{1m}^{-1}. Under the conditions of fast proton

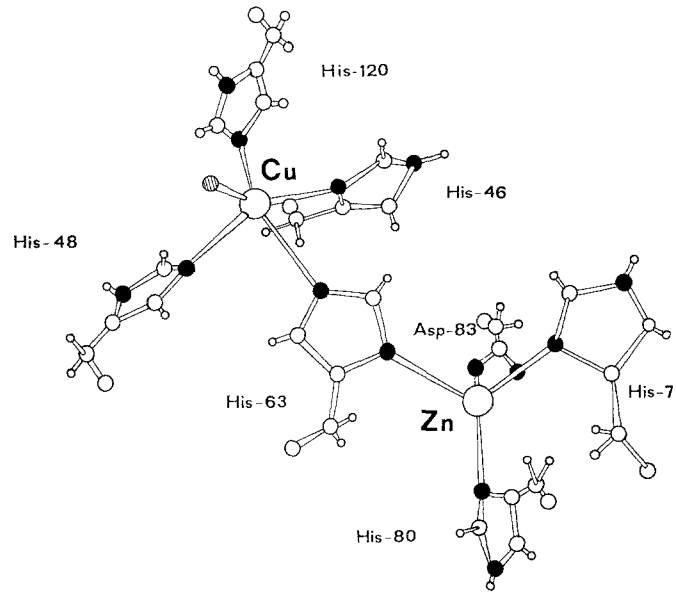

Fig. 1. Scheme of the metal sites in Cu_2Zn_2SOD. Copper is coordinated to four histidines and solvent exposed. Zinc is bound to three histidines and one aspartate residue. One histidine in the form of a negative ion bridges the two metal ions (Tainer et al. 1982)

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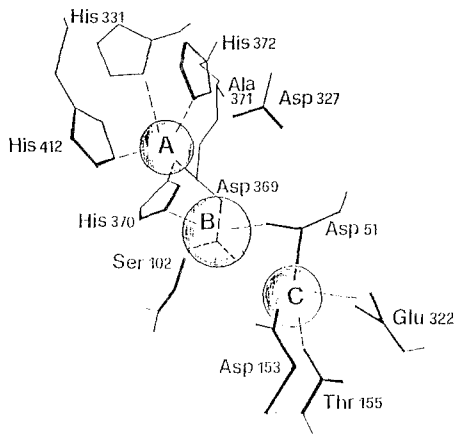

Fig. 2. Scheme of the metal sites in AP (Wyckoff 1983). A recent refinement (Wyckoff, personal communication) seems to indicate that Asp327 may be coordinated to the A-site metal in the place of His372

exchange between bulk solvent and sites close to the copper ion, this value depends on the number and distance of the exchangeable protons, on B, on the electronic relaxation time, τ_s, and on the copper nucleus-unpaired electron hyperfine coupling, A:

$$T_{1m}^{-1} = Gf(\tau_s, B, A)$$

where G is the geometric factor. Such equations have been derived in our laboratory (Bertini et al. 1985a, 1985b).

The electronic relaxation time of copper has now been determined and shown not to be affected by a second magnetically coupled copper ion, whereas it is affected by the coupling with cobalt(II) or nickel(II) ions. In the latter case the system is suitable for high-resolution NMR investigation.

Materials and methods

Alkaline phosphatase from *Escherichia coli* was isolated and purified according to the method reported by Applebury et al. (1979). The apoprotein was prepared through extensive dialysis against 2M $(NH_4)_2SO_4$ in 10 mM Tris pH 9 (Gettins and Coleman 1983). The metal derivatives were prepared by adding stoichiometric amounts of metal ions to the apoprotein, in water at pH 6.

Bovine liver superoxide dismutase was purchased from DDI (Mountain View). Human SOD and its mutants expressed in yeast were prepared as previously reported (Hallewell et al. 1987). The apoenzyme was obtained through dialysis against 10 mM EDTA in 50 mM sodium acetate pH 3.8 (McCord and Fridovich 1969). The Cu_2Co_2 derivative was prepared by addition of the stoichiometric amount of Co^{2+} solution to the apoprotein in acetate buffer pH 5.5 and then by slowly infusing the required amount of a solution of Cu^{2+} (Fee 1973).

The deuterated samples were prepared by repeated dilution with D_2O and concentration under nitrogen flow to a final D_2O content higher than 95%.

^{1}H-NMRD measurements were performed using the field-cycling relaxometer built at the IBM Laboratories (Yorktown Heights, NY) as previously described (Koenig and Brown III 1987).

^{1}H-NMR spectra were recorded at 90, 200, 300 MHz using a Bruker 90 CXP, a Bruker 200 MSL and a Bruker 300 CXP, respectively. The modified DEFT pulse sequence was used in order to suppress H_2O and bulk protein signals (Hochmann and Kellerhals 1980).

Results

The NMRD data

The water ^{1}H T_1^{-1} NMRD data on native copper-zinc SOD and some of its mutants are shown in Fig. 3 (Gaber et al. 1972). The data for the native protein have been interpreted with a τ_s of 2.8 ns and with a Cu–O distance of 0.25 nm if a single water molecule senses the copper ion (Bertini et al. 1985b). This value is consistent with that proposed on the basis of EXAFS stud-

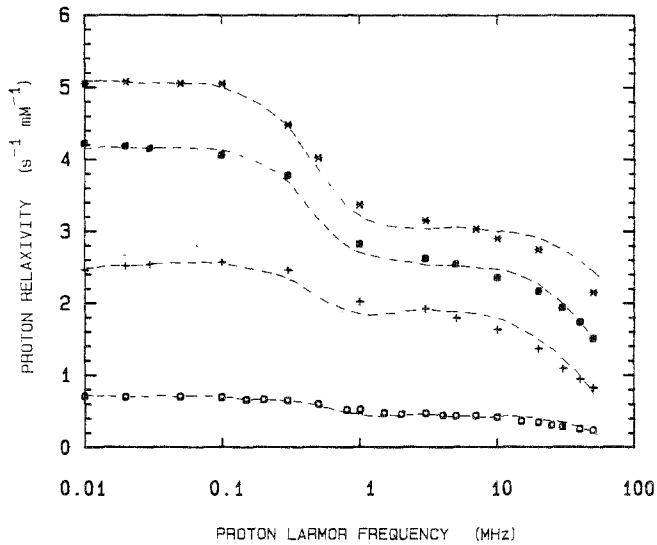

Fig. 3. Water ^{1}H T_1^{-1} profiles of: (●) Cu_2Zn_2SOD (Gaber et al. 1972), (+) Cu_2Cu_2SOD (Bertini et al. 1988), (□) Cu_2Zn_2(Ile-137)SOD (Banci et al. 1989a), (*) Cu_2Zn_2(Ser137)SOD (Banci et al. 1990b)

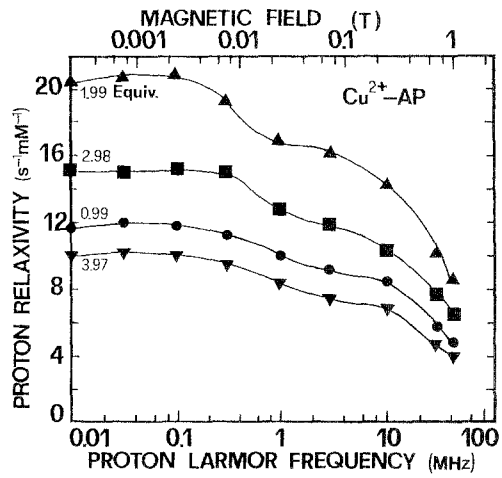

Fig. 4. Water ^{1}H T_1^{-1} profiles of Cu_2E_2AP (Bertini et al. 1989) at 298 K, pH 6, for Cu(II)/protein ratios of: (●) 0.99, (▲) 1.99, (□) 2.98 and (▼) 3.97 (Bertini et al. 1989)

ies (Blackburn et al. 1987). Although the amount of water in the cavity can be changed according to the nature of the mutated residue, the τ_s value is always essentially the same.

The profile of Cu_2E_2AP is reported in Fig. 4. In this case, τ_s is 4 ns and, if a single water is sensing the copper ion, the Cu-O distance is 0.20 nm.

In the case of Cu_2Cu_2SOD antiferromagnetic coupling occurs, with an energy separation between the ground $S=0$ and excited $S=1$ state of 52 cm^{-1} (Fee and Briggs 1975). The fitting of the experimental data provides $\tau_s=4.2$ ns and a Cu-O distance of 0.23 nm (Bertini et al. 1988). It appears that nothing has changed, within experimental error, with respect to the native protein, despite the profile of Cu_2Cu_2SOD being a half that of the native protein. This is fully understood on the basis that the magnetically active $S=1$ state spends half of the time in the $M_s=0$ level which is not efficient for relaxation (Bertini et al. 1988; Owens et al. 1986). We believe that τ_s at the high magnetic fields of these measurements essentially corresponds to the longitudinal electronic relaxation time T_{1e} (Bertini and Luchinat 1986).

In the case of AP, the affinity of copper for the B site when the A site is occupied is low (Bertini et al. 1989). From the titration of apo-AP with copper(II) (Fig. 5), it appears that the introduction of copper in the B site reduces the water ^1H T_1^{-1} values more than expected, just as if a water molecule were prevented from fast exchange or removed from coordination (Bertini et al. 1989). The τ_s value seems not to be affected. From both these copper proteins, we learn therefore that the longitudinal electronic relaxation time is not changed upon magnetic coupling with the same copper ion, whereas from EPR we know that T_{2e} is slightly smaller in the dimer (Valentine et al. 1979).

When cobalt(II) is added to Cu_2E_2SOD (Fig. 6) or Cu_2E_2AP (Fig.7) the water ^1H T_1^{-1} values dramatically decrease and τ_s is shortened. In the case of SOD the final profile has not been obtained, probably on account of the slow rate of cobalt uptake.

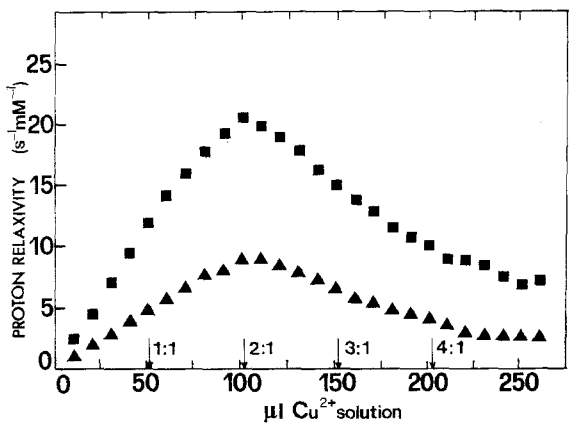

Fig. 5. Water proton relaxivity at (■) 0.01 MHz and (▲) 40 MHz of apo-AP with increasing amounts of added copper(II) ions. The points corresponding to 1:1, 2:1, 3:1 and 4:1 mol copper(II)/mol protein are indicated on the x axis (Bertini et al. 1989)

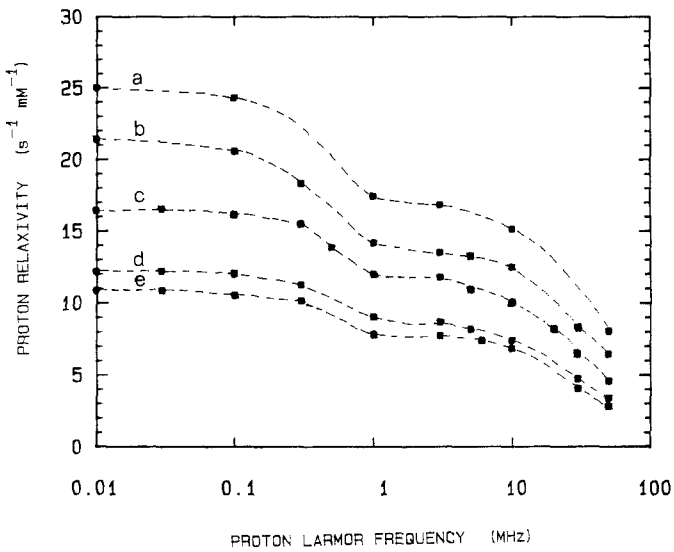

Fig. 6. Water proton relaxivity (expressed per mol protein) of (a) Cu_2E_2SOD and (b-e) Cu_2E_2SOD with increasing amounts of added cobalt(II) ions: 0.7 (b), 1.3 (c), 2.0 (d) and 2.7 (e) mol cobalt(II)/mol protein

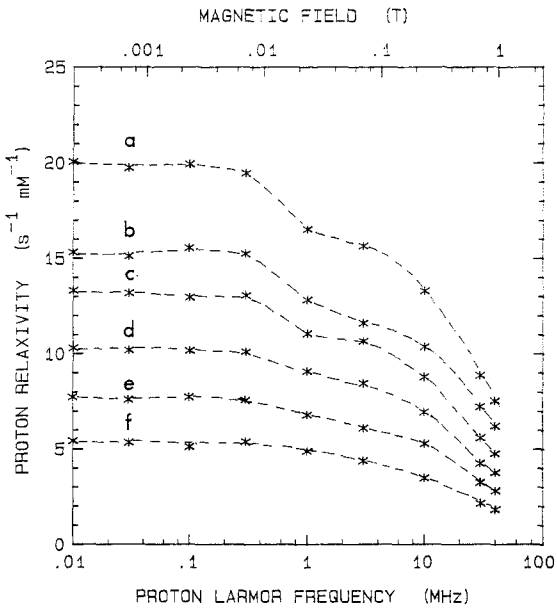

Fig. 7. Water proton relaxivity (expressed per mol protein) of: (a) Cu_2E_2AP and (b-f) Cu_2E_2AP with increasing amounts of added cobalt(II) ions: 0.4 (b), 0.8 (c), 1.2 (d), 1.6 (e) and 2.0 (f) mol cobalt (II)/mol protein (Banci et al. 1988)

High-resolution ^1H-NMR experiments

The occurrence of magnetic coupling between copper(II) and cobalt(II) reduces the electronic relaxation times of the former. This is because cobalt(II) has short electronic relaxation times and provides efficient mechanisms for copper relaxation as well (Bertini and Luchinat 1986). The effect of a fast-relaxing metal ion on a slow-relaxing metal ion can be determined in a perturbative way as long as the interaction energy is

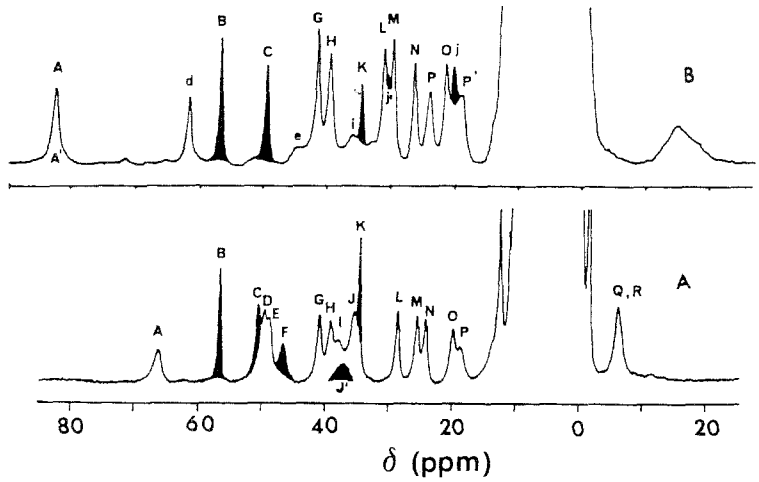

Fig. 8. 300-MHz room-temperature NMR spectra of (A) Cu_2Co_2SOD and (B) Cu_2Ni_2SOD. The shaded signals disappear in D_2O. The labels indicate the correspondence between the two derivativs (Ming et al. 1988)

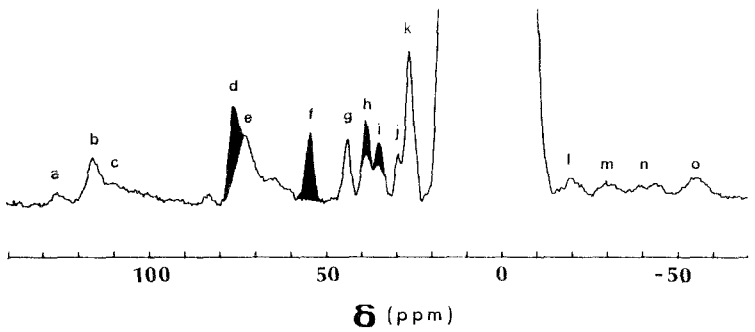

Fig. 9. 90-MHz room-temperature NMR spectra of $Cu_2Co_2Mg_2AP$ in H_2O. The shaded signals disappear in D_2O. The samples are unbuffered at pH ≈ 6 (Banci et al. 1988)

smaller than the electronic relaxation rate of the fast-relaxing metal ion (Banci et al. 1990a). This is not the case here as the magnetic interaction energy is quite large. However, from the NMRD profile and the NMRD studies shown below, it appears that the electronic relaxation times of copper(II) decrease by two orders of magnitude. Under these circumstances the ^{1}H-NMR spectra can provide reasonably sharp signals for the protons of the histidines bound to both copper and cobalt ions. This is an important result for providing a tool to monitor structural variations in the active cavities.

In Fig. 8A the ^{1}H-NMR spectrum of Cu_2Co_2SOD is shown and in Table 1 the assignment is reported (Banci et al. 1987). The shaded lines are due to exchangeable protons that have been assigned to histidine NH protons. The full assignment has been reached through ^{1}H-NOE experiments (Banci et al. 1989b). In Fig. 9 the spectrum of $Cu_2Co_2Mg_2AP$ is reported (Banci et al. 1988). Again, the shaded signals are assigned to exchangeable protons, at least two of which are due to histidine NHs. The protein is too large to be studied with high-field NMR instruments and NOEs have not been measured. The assigment is therefore only tentative (Bertini et al. 1988).

Since tetrahedral nickel(II) is expected to have short electronic relaxation times, we have also investigated Cu_2Ni_2SOD (Fig. 8). The ^{1}H-NMR signals are sharper than in the case of the cobalt analog (Ming et al. 1988). A correspondence of the signals of the copper(II) domains in the two systems has been established.

Table 1. 300 MHz ^{1}H NMR shift at 303 K for the isotropically shifted signals in the Cu_2Co_2SOD derivative with their assignment

Signal	δ (ppm)	Assignment
A	66.2	His-61 Hδ2[a,b]
B	56.5	His-118 Hδ1[a]
C	50.3	His-44 Hε2[a]
D	49.4	His-78 Hδ2[b]
E	48.8	His-69 Hδ2[b]
F	46.7	His-78 Hε2 (His-69 Hε2)[b]
G	40.6	His-44 Hδ2[a]
H	39.0	His-118 Hε1[a]
I	37.4	Asp-81 Hβ1 (Asp-81 Hβ2)[b]
J′	35.6	Asp-81 Hβ2 (Asp-81 Hβ1)[b]
J	35.4	His-69 Hε2 (His-78 Hε2)[b]
K	34.5	His-46 Hδ1[a]
L	28.4	His-46 Hδ2[a]
M	25.3	His-44 Hε1[a]
N	24.1	His-118 Hδ2[a]
O	19.6	His-46 Hε1[a]
P	18.7	His-44 Hβ1[a]
Q	−6.2	His-69 Hβ2[a]
R	−6.2	His-44 Hβ2[a]

[a] Banci et al. (1989b); [b] Banci et al (1987)

Concluding remarks and perspectives

When the electronic relaxation times of copper(II) or any other ion are long, EPR, ENDOR (Hüttermann and Kappl 1987) and ESE (Fee et al. 1981; Tsvetkov and Dikanov 1987) measurements are easily performed

as well as NMRD (Banci et al. 1985). In the latter case, the actual values of the electronic relaxation times and the existence of closely located exchangeable protons can be discovered. Upon magnetic coupling with another equal ion, the nuclear T_1^{-1} values are halved while τ_s does not change. When magnetic coupling with a fast-relaxing metal ion occurs, then τ_s dramatically decreases and high-resolution NMR studies can be performed. Even a relatively small magnetic coupling can considerably reduce τ_s. This can be developed into a general tool for investigation via NMR systems which would be unsuitable without magnetic coupling.

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