Magnetic Relaxation of Solvent Protons by Cu^{2+}- and VO^{2+}-Substituted Transferrin: Theoretical Analysis and Biochemical Implications

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ABSTRACT: Measurements of the magnetic field dependence of the longitudinal nuclear magnetic relaxation rates of solvent protons (NMRD profiles) in solutions of paramagnetic proteins have contributed significantly to the elucidation of the physical biochemistry of a number of metalloprotein systems. In many cases, NMRD profiles were used as indicators of chemical state, both static and dynamic [cf. Brewer, C. F., Brown, R. D., III, & Koenig, S. H. (1983) *J. Biomol. Struct. Dyn. 1*, 961–997], in part because a proper theoretical description of the data, with realistic assumptions for a model system, was computationally intractable. This has been particularly true for Cu^{2+}–protein complexes, attributable in part to the $S = {}^1/_2$ ground-state configuration of the Cu^{2+} ions; significant progress in interpreting such data has been made only recently [Bertini, I., Briganti, F., Luchinat, C., Mancini, M., & Spina, G. (1985) *J. Magn. Reson. 63*, 41–55]. We report NMRD profiles for solutions of Cu^{2+}- and VO^{2+}-substituted human transferrin, both $S = {}^1/_2$ ions, as well as computations that include the effects of the anisotropic hyperfine interactions of the paramagnetic ions with their respective nuclei. The description of the data that results from these computations is quite good, sufficiently so that one can say with confidence that the protons that contribute to the relaxation are rather distant (~3.5 Å) from the ions and in rapid exchange ($\sim 10^8$ s^{-1}) with solvent. A possible view, consistent with what is known of the biochemistry of these substituted transferrins, is that relaxation occurs in the second coordination sphere: the exchanging entity is a water molecule hydrogen bonded to a donor atom of the metal ion complex.

Well before technology had advanced to the point where high-resolution, chemical shift, proton nuclear magnetic resonance (NMR)[1] spectroscopy had become possible, among the earliest applications of NMR was to the study of the ligand structure of hydrated transition-metal ions in solution. Questions regarding the number and exchange rate of coordinated water molecules, and the mechanisms of their replacement by small solute anions, could be addressed by measurements of the magnetic relaxation rates of solvent protons. At an early date, Bloembergen et al. (1948) proposed a theory of relaxation of solvent protons by paramagnetic ions, noted that the rates were dependent on magnetic field strength, and stressed the importance of solvent viscosity and its variation with temperature. A more realistic model of the aquo ion was considered by Kubo & Tomita (1954) and Solomon (1955) followed by experimental investigations by a number of groups, including a thorough analysis of the temperature dependence of relaxation rates in solutions of Co^{2+}, Cu^{2+}, Mn^{2+}, and Gd^{3+} (Bernheim et al., 1959) and extensive data on the magnetic field dependence of the relaxation rates of solutions of Co^{2+}, Cr^{3+}, Cu^{2+}, Gd^{3+}, Mn^{2+}, Nd^{2+}, and Ni^{2+} (Nolle & Morgan, 1957; Morgan & Nolle, 1959). In this early work, the refinement of relaxation theory, including insights into relaxation mechanisms, went hand-in-hand with the chemical information that was forthcoming from the experiments themselves.

As instrumentation improved, studies on small solute molecules shifted toward high-resolution proton spectroscopy, and relaxation studies were used more and more for probing the structure of the paramagnetic sites of metalloproteins [cf. Brewer et al. (1983)], the earliest of which were Fe^{3+}–protein complexes [cf. Koenig & Brown (1984) for a review and extensive references]. The initial measurements were made at one value of magnetic field strength, but it soon became clear (Wishnia, 1960) that ambiguities in interpretation of the data might only be resolved, if at all, by measurements of solvent proton relaxation rates over a wide range of magnetic field strength (NMRD profiles).

The first NMRD profiles reported for protein solutions were for saturated native (Fe^{3+}) transferrin (TFN) (Koenig & Schillinger, 1969) and Cu^{2+}–TFN, both in the presence and absence of HCO_3^- (Gaber et al., 1970). It was apparent at the time that the theory, with the model assumptions that worked so well for describing the NMRD profiles of these ions in solution, was inadequate to describe the data for solutions of protein complexes of these ions. Cu^{2+}–protein complexes were particularly intractable, judging from the initial work on Cu^{2+}–TFN (Gaber et al., 1970) and later studies of a wide range of Cu^{2+}–protein complexes (Gaber et al. 1972; Koenig & Brown, 1973). The problem is that protein complexes in solution rotate slowly compared to the smaller aquoions, so that the anisotropic structure of the ligand fields of the complexed ions is not averaged out in solutions of protein complexes as it is for small paramagnetic ions, including complexes of these ions with solute anions. This enormously complicates the application of relaxation theory to solutions of paramagnetic metalloproteins.

Recently Bertini and his colleagues (Bertini et al., 1984a, 1985a–c), following the pioneering work of Lindner (1965),

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[1] Abbreviations: NMR, nuclear magnetic resonance; NMRD, nuclear magnetic relaxation dispersion, in which the solvent longitudinal proton magnetic relaxation rate is measured as a function of the applied static magnetic field; TFN, transferrin with unspecified metal ion content; EPR, electron paramagnetic resonance.

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have developed equations that take into account all the parameters of the electronic spin Hamiltonian that are needed to give a good description of the electron-nucleus dipolar coupling. In particular, they included the hyperfine interactions of metal ions with their own nuclei for ions such as Cu^{2+}, for which the electronic relaxation rates are very low and, in large part, result from this coupling (Bertini et al., 1985a; Bertini & Luchinat, 1985a). It had been suggested in the initial work on Cu^{2+}–TFN that hyperfine interactions could be the major complicating factor (Gaber et al., 1970), but it is only now that a quantitative comparison of relaxation theory with Cu^{2+}–TFN has been made.

In the present paper, the methods of Bertini et al. (1985a) are applied to Cu^{2+}–TFN, which has a single hole in the d shell, and to VO^{2+}-transferrin, which has a single d electron. The electronic states of the two ions are similar. Both have an $S = {}^1/_2$ ground state, long electronic relaxation times, and relatively large electron-nuclear couplings. What is different is that an oxygen occupies a ligand position of VO^{2+}–TFN that is presumably occupied by H_2O or OH^- in Cu^{2+}–TFN; the five other ligands of the metal ions are believed to be HCO_3^- and protein-donated histidines and tyrosines, though not necessarily disposed identically in the two derivatives [cf. Chasteen (1983) and Bertini et al. (1984b), and references therein]. Thus, solvent accessibility to the two paramagnetic complexes ought to be grossly different, as a result of which the amplitudes of the NMRD profiles of Cu^{2+}–TFN and VO^{2+}–TFN might be expected to differ substantially, being less for the vanadyl complex. Despite the preceding, to the extent that the profiles of Cu^{2+}– and VO^{2+}–TFN solutions differ significantly, they do so only quantitatively, and VO^{2+}–TFN is the better relaxing complex. The purpose of the present paper is to reconcile these relaxation data, which can now be confidently handled with reasonable precision within the extended framework of relaxation theory, with the known biochemical structure and properties of these two substituted transferrins.

Materials and Methods

Materials. Demetallized (apo) TFN from Sigma Chemical Co. was dialyzed against 0.1 M citrate buffer of pH 4.5, then against 0.1 M perchlorate, and finally against freshly doubly distilled water (Bates & Schlabach, 1973). This procedure removes possible protein-bound chelate. The metal derivatives were obtained by titrating the apo-TFN with $CuSO_4$ and $VOSO_4$ in the presence of 0.03 M HCO_3^- near pH 8. Co^{3+}–TFN, used as a diamagnetic blank instead of apo-TFN (Koenig & Schillinger, 1969), was prepared by titration with $CoSO_4$, with subsequent oxidation of the metal ion in situ with hydrogen peroxide. The titration was followed spectrophotometrically by using the known metal–ligand charge-transfer bands to confirm the expected 2:1 stoichiometry of the saturated protein. Excess, nonspecifically bound, metal was removed by dialysis. HCO_3^- was present in all samples, in equilibrium with atmospheric CO_2.

NMRD Measurements. Longitudinal (spin–lattice) relaxation rates of solvent protons were measured by using a field-cycling relaxometer referenced previously [cf. Brown et al. (1977)]. Sample volumes were routinely about 0.6 mL. Reproducibility of the data for a given sample was generally better than ±1%, and the time required to obtain a single data point was typically 2–3 min. Magnetic field values are given primarily in units of the Larmor precession frequency of protons in that field.

Comparison of Data and Theory. The equations that describe $1/T_{1para}$, the paramagnetic contribution to the relaxation rate, have been considered in detail in many publications [cf. Koenig & Brown (1984) for a recent summary]; those that are particularly germane to the present work are

$$\frac{1}{T_{1para}} = \frac{[M]}{[H_2O]} \frac{1}{T_{1M} + \tau_M} \tag{1}$$

where T_{1M} is the longitudinal relaxation time of a proton of a moiety coordinated to the metal ion and τ_M is its residence lifetime. [M] is the concentration of metal ions. The theory for T_{1M}, as altered here to take into account A, the anisotropic hyperfine interaction of the paramagnetic ions with their nuclei, can be written as

$$\frac{1}{T_{1M}} = \frac{3.29 \times 10^{-32}}{r^6} \left[7F(H_0, \tau_c, A, \theta) + \frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} \right] \ (s^{-1}) \tag{2}$$

as long as

$$\omega_S/\omega_I \gg A/(\hbar\omega_S) \gg 1 \tag{3}$$

Here F is a function that depends on the magnetic field strength H_0 and on a correlation time τ_c; it has to be computed numerically for each value of its arguments (Bertini et al., 1985a). The proton ion separation, assumed the same for all protons, is given by r, and θ is the angle between the metal proton direction and the principal axis of the A tensor, assumed uniaxial in the present case on the basis of EPR data.

For ions such as Cu^{2+} and VO^{2+}, τ_c depends on the electronic relaxation time τ_S and on τ_M and may be dominated by one or the other. In fitting eq 2 to the data, τ_c has been assumed independent of H_0, which would be true if τ_M dominates τ_c and a very good approximation if not.

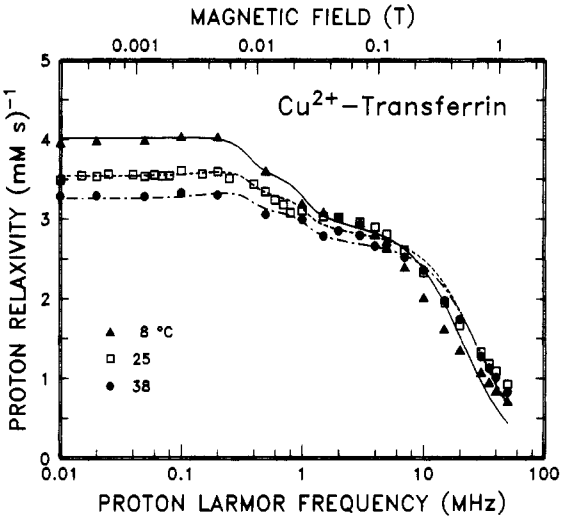

FIGURE 1: Proton NMRD profiles for water solutions of saturated Cu^{2+}–transferrin (1.68 mM in Cu^{2+}), at pH, for three temperatures. The results are expressed in relaxivity units, the increment in relaxation rate per millimolar of added paramagnetic ion (not per macromolecule). The curves through the data points result from a least-squares comparison of data and theory by using $I = {}^3/_2$ for the nuclear spin, $A_\parallel = 168 \times 10^{-4}$, $A_\perp = 20 \times 10^{-4}$ cm^{-1}, and the values for G (related to r), τ_c, and θ given in Table I.

Results

Cu^{2+}–Transferrin. Figure 1 shows the $1/T_{1para}$ NMRD profiles of Cu^{2+}–TFN at three temperatures. The results are expressed as relaxivity $[(1/[M])T_{1para}]$, the increment in the relaxation rate per millimolar of added paramagnetic ion (not per macromolecule). In terms of eq 2, the data at fields above

Table I: Results of Least-Squares Comparisons of Measured NMRD Profiles with Relaxation Theory for Aqueous Solutions of Cu^{2+}- and VO^{2+}-Transferrin

sample	temp (°C)	$G^a \times 10^{-44}$ (cm^{-6})	τ^c (ns)	θ (deg)
Cu^{2+}-TFN	8	5.48	7.58	45.5
	25	7.36	5.70	50.0
	38	7.47	5.40	51.7
VO^{2+}-TFN	8	7.95	22.4	26.7
	25	7.81	19.2	29.5
	38	4.74	20.5	23.6

[a] $G = \sum_i (n_i/r_i^6)$, where n_i is the number of (equivalent) protons interacting with the metal ion at distance r_i. For a single water molecule in the usual coordination geometry, r = 2.8 Å and $G = 4.15 \times 10^{45}$ cm^{-6}.

~1 MHz are described by the second term in the bracket, and the half-point, the field at which this portion decreases to a relaxivity of ~1.5 from ~3 $(mM\ s)^{-1}$, corresponds to $\omega_I\tau_c = 1$ and $\tau_c = 6 \times 10^{-9}$ s for the 25 °C profile. This value is well within the range of τ_S values reported for all copper proteins investigated so far (Bertini & Luchinat, 1985b). Although this does not rule out a significant contribution of τ_M to τ_c, the inference we draw is that τ_c is dominated by τ_S. The slight variation of τ_c with temperature is consistent with this point.

From the preceding estimate of τ_c, the measured relaxivity at $\omega_I\tau_S = 1$, and eq 1 and 2, one can readily estimate r = 3.9 Å, corresponding to the relatively large value of 3.0 Å for the Cu–O separation. (F, eq 2, makes negligible contribution at this field.) The detailed application of theory, using the value $A_\parallel = 167 \times 10^{-4}$ cm^{-1} and $A_\perp$ ranging from 0 to 20×10^{-4} cm^{-1} (Froncisz & Aisen, 1982), is shown by the smooth curves, which were drawn through 50 computed points (20×10^{-4} cm^{-1} is an upper limit for $A_\perp$, as estimated from the line width of a particular feature in the $g_\perp$ region of the EPR spectrum.) The fit gives r = 3.7 Å, assuming a single water molecule with its protons equidistant from the Cu^{2+}, corresponding to the Cu–O bond length of 2.9 Å. (This computed value is somewhat lower than the estimate above, which depended on finding the midpoint of a dispersion that is not well defined.) Assuming a single proton gives r = 3.3 Å. Additional results are in Table I.

VO^{2+}-Transferrin. Figure 2 shows the $1/T_{1para}$ NMRD profiles of VO^{2+}-TFN at three temperatures. Qualitatively, the data are similar to those of Cu^{2+}-TFN (Figure 1). Quantitatively, the relaxivities are 2–3-fold greater; the temperature variation is greater, though the sign is the same, and there appears to be more resolved structure in the field range 1–5 MHz. The fit to these data (Figure 2) using the reported values for the components of $A_\parallel = 170 \times 10^{-4}$ and $A_\perp = 60 \times 10^{-4}$ cm^{-1} (White & Chasteen, 1979) is quite satisfactory, though the calculations tend to exaggerate the structure noted above. The results of the fit are in Table I. The derived values for τ_c are 2–3 times longer than for the copper derivative, consistent with the τ_s values of VO^{2+} compounds.

Discussion

In the past, the analysis and interpretation of proton NMRD profiles of Cu^{2+}–proteins has been particularly difficult because of the wide variety of profiles observed (Koenig & Brown, 1973; Bertini et al., 1985a; Bertini & Luchinat, 1985a), all of which deviate substantively from the forms expected on the basis of theory with the classic, simplifying assumptions. The presumption was that the $S = {}^1/_2$ ground state of the Cu^{2+} ions, which contributed to a long τ_S [cf. Koenig & Brown (1984)], and perhaps the tendency to square-planar coordination, which would give a short τ_M for an apical water, contributed to the problem. Analogous results are found here

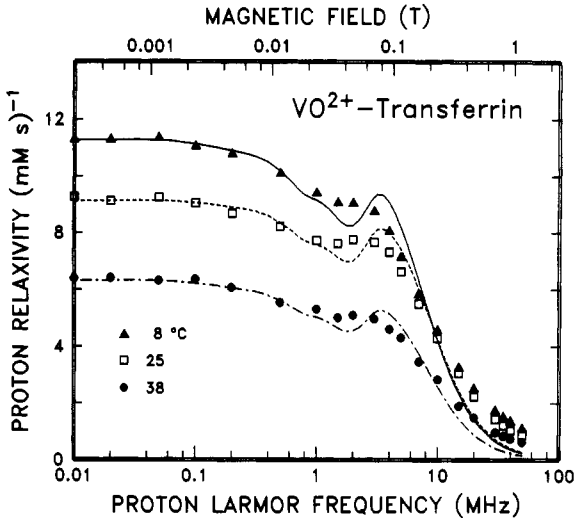

FIGURE 2: Proton NMR profiles for water solutions of saturated VO^{2+}–transferrin (1.64 mM in VO^{2+}), at pH 8, for three temperatures. The results are expressed in relaxivity units, the increment in relaxation rate per millimolar of added paramagnetic ion (not per macromolecule). The curves through the data points result from a least-squares comparison of data and theory by using $I = {}^7/_2$ for the nuclear spin, $A_\parallel = 170 \times 10^{-4}$, $A_\perp = 60 \times 10^{-4}$ cm^{-1}, and the values for G (related to r), r, τ_c, and θ given in Table I.

for VO_2^{+}-TFN, in which the vanadyl ion also has an $S = {}^1/_2$ ground state; the NMRD profiles of Cu^{2+}- and VO^{2+}-TFN are very similar to each other and very different from the NMRD profiles of most other metal ion–protein complexes, supporting the notion of a special character for the relaxation properties of the $S = {}^1/_2$ ground states.

It is particularly satisfying that the theory of relaxation, as augmented to include anisotropic hyperfine interactions with the nuclei of the paramagnetic ions (Bertini et al., 1985a), is as successful as it is here in describing the measured profiles of the two transferrin derivatives. The major result, true for both, is that the observed proton–metal ion interaction is rather weak, equivalent to a single proton, in rapid exchange, coordinated rather far, about 3.3 Å, from the paramagnetic ions. This long distance, and the similarity of the behavior of both profiles, when the vanadium presumably has no solvent-donated ligand and the Cu^{2+} presumably does, suggests that relaxation occurs in the second coordination sphere of the metal ions in both cases. Thus, the proton of a water molecule possibly hydrogen bonded to the vanadyl oxygen, or to the solvent-donated ligand of the Cu^{2+}, could make the predominant contribution to the observed NMRD profiles. The possibility that the second sphere water can, instead, interact via hydrogen bonding with the synergistic bicarbonate ion, or even with an oxygen of a coordinated tyrosine, should also be considered; removal of bicarbonate is known to alter the profile drastically (Gaber et al., 1970).

Though the agreement of data and theory is very good, there remain small, but systematic, differences, particularly in the data for VO^{2+}-TFN, above about 2 MHz. We attribute this to the implicit assumption that τ_c is independent of H_0, which is not strictly true. However, the range of variation of τ_c in the present case, as has been argued for Cu^{2+}–proteins previously (Koenig & Brown, 1973), is expected to be rather small, being limited at high fields by the relatively short τ_M, unlike the situation for, say, Mn^{2+}–protein complexes [cf. Koenig & Brown (1984)], for which τ_M is longer and the field dependence of τ_c dominates the NMRD profiles.

A second coordination configuration has been invoked previously to explain relaxation in fluoromethemoglobin

(Koenig et al., 1981) and Fe^{3+}–TFN (Koenig & Brown, 1983), in which the solvent-donated ligand is OH^-. As in the present work, a relatively distant and rapidly exchanging water was needed to explain those data. It is not established yet whether OH^- or H_2O is the solvent-donated ligand for Cu^{2+}–TFN, nor is it even clear that the protein ligands of Cu^{2+}– and VO^{2+}–TFN are organized identically; these are biochemical questions that remain to be answered. What is now established, however, is that any biochemical model must accord with the implications of the NMRD profiles, which can now be analyzed quantitatively, and with confidence, for these two transferrin derivatives.

Registry No. Cu, 7440-50-8; VO^{2+}, 20644-97-7.

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