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Gadolinium(III) as a Paramagnetic Probe for Proton Relaxation Studies of Biological Macromolecules. Binding to Bovine Serum Albumin*

Jacques Reuben†

ABSTRACT: An investigation of the applicability of proton relaxation methods in studying metal ion binding to biological macromolecules using gadolinium(III) as a paramagnetic probe has been carried out. The binding of Gd(III) to bovine serum albumin has been studied. The longitudinal proton relaxation rate of water due to the paramagnetic Gd(III)–bovine serum albumin complex is enhanced relative to the ion in solution. Using the enhancement as an analytical parameter it was found that bovine serum albumin has four binding sites for Gd(III) with an apparent dissociation constant of 1.3×10^{-4} M at 300°K. Conclusions regarding the mechanism of enhancement have been drawn from measurements at different frequencies and temperatures. The correlation time for the electron–nuclear dipolar interaction is dominated by the rotation of the complex for the aquo and cacodylate complexes of Gd(III), whereas for the Gd(III)–bovine serum albumin complex it is mainly the electron spin relaxation time.

The use of transition-metal paramagnetic ions as probes for magnetic resonance studies of biological macromolecules is well documented (Mildvan and Cohn, 1970; Cohn and Reuben, 1971). In this paper we present results of an investigation aimed to establish the extent of applicability of magnetic resonance methods in studies of the macromolecular environment of ions using a member of the lanthanide series, gadolinium(III), as the paramagnetic probe.

Inhibition of enzymatic reactions (Holten *et al.*, 1966, and references cited therein) and of calcium transport across membranes (Mela, 1969) by trivalent lanthanides has been reported. More recently it has been found that lanthanides may act as cofactors in the activation of trypsinogen by trypsin (Darnall and Birnbaum, 1970).

Among the paramagnetic transition-metal ions manganese(II) has been found to be most suitable as a probe for magnetic relaxation studies of macromolecular systems. From the magnetic resonance point of view its "analog" in the lanthanide series is gadolinium(III) which is also an *S*-state ion with high electronic spin ($S = 7/2$), relatively long electron relaxation time, and labile hydration sphere. Therefore gadolinium(III) was chosen for this investigation. Bovine serum albumin is a readily available protein known to bind metal ions (*cf.*, *e.g.*, Mildvan and Cohn, 1963). Preliminary experiments have shown that the longitudinal proton relaxation rate in aqueous solutions of $GdCl_3$ is enhanced in presence of albumin suggesting that gadolinium does bind to this protein.[1] Therefore bovine serum albumin was chosen as the macromolecule for this study. In conjunction with the albumin study and for comparison also studied were solutions of $GdCl_3$ in absence and in presence of tetramethylammonium cacodylate (dimethyl arsinate), which was used as buffer.

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[1] The preliminary experiments were carried out in collaboration with Dr. F. Lantelme.

Experimental Section

Longitudinal proton relaxation times, T_1, were measured at 8.13, 24.3, and 60.0 MHz by the 180–90° null method of Carr and Purcell (1954). The accuracy of measurements is estimated to be ±2% of the T_1 values. The sample temperature was controlled to within ±0.4°. The sample volume was in the range 0.08–0.12 ml.

The paramagnetic contribution to the relaxation rate, $1/T_{1p}$, is defined as

$$1/T_{1p} = 1/T_1 - 1/T_1^\circ \quad (1)$$

where T_1 is the observed relaxation time in presence of paramagnetic ions and T_1° is the relaxation time of an otherwise similar solution but in absence of paramagnetic ions. In dilute (aqueous) solutions $1/T_{1p}$ is given by (O'Reilly and Poole, 1963; Luz and Meiboom, 1964)

$$1/T_{1p} = P_M/(T_{1M} + \tau_M) \quad (2)$$

where T_{1M} and τ_M are, respectively, the relaxation time and the mean residence time of the nucleus in the paramagnetic complex and $P_M = n[M]/55.5$, n being the number of water molecules in the first coordination sphere of the metal ion of concentration [M] and 55.5 is the molarity of water. It is seen from eq 2 that $1/T_{1p}$ is proportional to the concentration of the paramagnetic ion. Therefore T_1 was measured at several concentrations of $GdCl_3$ in absence (pH 3) and presence (pH 6.3) of 0.05 M tetramethylammonium cacodylate buffer and values of $1/T_{1p}$ at 10^{-4} M $GdCl_3$ were evaluated by interpolation from a plot of $1/T_{1p}$ against concentration. In presence of albumin at least two types of paramagnetic species exist in equilibrium: the protein-bound and free ions, and therefore $1/T_{1p}$ in this case was obtained from one measurement of T_1 and one of T_1°.

Since lanthanide hydroxides precipitate near neutral pH a buffer solution 0.05 M in tetramethylammonium cacodylate at pH 6.3 was used. Crystallized bovine serum albumin, obtained from Pentex, Inc., was dissolved in the buffer. Insoluble material was removed by centrifugation. The concentration was determined from the absorbance at 280 mμ using 0.66 as the absorbance of 1 mg/ml per cm and a molecular weight of 69,000 (Cohn *et al.*, 1947). Solutions of $GdCl_3$ were prepared from a 1 M stock solution made by reacting Gd_2O_3 (from Alfa Inorganics) with dilute hydrochloric acid. To maintain ionic strength all solutions were made 0.1 M in tetramethylammonium chloride.

Results

Binding of Gd(III) to Albumin. The number of binding sites of albumin for Gd(III) and the dissociation constant of the Gd(III)–albumin complex were determined from the water proton relaxation rates at a constant concentration of Gd(III) and varying amounts of the protein. The approach is similar, to some extent, to that previously used in a study of manganese binding to albumin (Mildvan and Cohn, 1963). It is convenient to use the enhancement of the relaxation rate, ϵ^*, which is defined as

$$\epsilon^* = \frac{1/T_{1p}^*}{1/T_{1p}} \quad (3)$$

where $1/T_{1p}^*$ is the observed paramagnetic contribution to the relaxation rate in presence of protein and $1/T_{1p}$ is the same but in absence of protein. The enhancement observed in a given sample is a sum of the contributions due to the two paramagnetic species present in solution

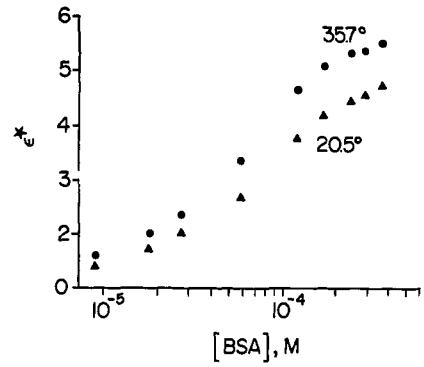

FIGURE 1: The enhancement, ϵ^* at 24.3 MHz, of the paramagnetic contribution to the longitudinal proton relaxation rate of water due to Gd(III) as a function of albumin concentration at two temperatures. The Gd(III) concentration is 2.1×10^{-4} M. The solutions are 0.05 M in cacodylate buffer at pH 6.3.

$$\epsilon^* = M_f/M_t + \epsilon_b M_b/M_t \quad (4)$$

where M_f is the concentration of the unbound (free) metal ions, M_b is that of ions bound to the protein, $M_t = M_f + M_b$, ϵ_b is the enhancement of the bound form, and the enhancement of the free ions is unity by definition. A plot of ϵ^* at 24.3 MHz against the total protein concentration at two temperatures is shown in Figure 1. As expected, ϵ^* approaches 1 at low protein concentrations where M_f is close to M_t. At high protein concentrations ϵ^* approaches asymptotically ϵ_b since $M_b \approx M_t$. Note that the enhancement is calculated with respect to a solution of $GdCl_3$ in the buffer and the cation is in the form of a Gd(III)–cacodylate complex (*vide infra*).

Assuming that there are n' independent and equivalent binding sites for the metal ion on the protein molecule the dissociation constant, K_D, is given by

$$K_D = M_f(n'P_t - M_b)/M_b \quad (5)$$

where P_t is the total protein concentration. Equation 5 may be rearranged to give the Scatchard (1949) form

$$\bar{\nu}/M_f = n'/K_D - \bar{\nu}/K_D \quad (6)$$

where $\bar{\nu} = M_b/P_t$.

The results were analyzed in the following manner. Equation 5 was used to calculate concentrations of free and bound Gd(III) for a series of K_D and n' values and ϵ_b was calculated from eq 4 using the experimental ϵ^*. For n' being 3, 4, 5, and 6 the values of ϵ_b thus obtained were constant to within ±5%. An average value of ϵ_b was used with the experimental ϵ^* to calculate concentrations from eq 4, which were then used to construct Scatchard plots ($\bar{\nu}/M_f$ *vs.* $\bar{\nu}$, see eq 6). The plots at two temperatures are shown in Figure 2. From the intercept on the abscissa the number of binding sites is found to be four. Measurements at several temperatures gave the same result for the number of binding sites. The apparent dissociation constants, obtained from the ratio of the intercept on the abscissa to that on the ordinate of the Scatchard plot, are

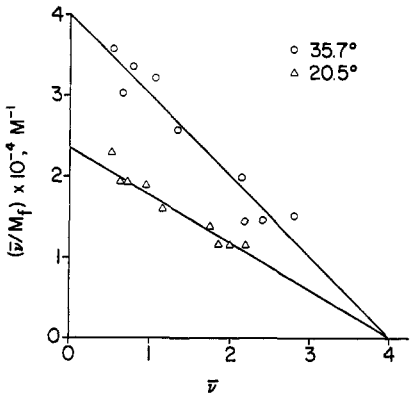

FIGURE 2: Scatchard plots (eq 6) for Gd(III) binding to albumin at two temperatures. The plots were constructed from the data on Figure 1 (see text).

plotted against the inverse absolute temperature in Figure 3. These are apparent dissociation constants since "free" Gd(III) exists as the cacodylate complex. At 300°K, $K_D = 1.3 \times 10^{-4}$ M. For comparison, at the same pH a tight binding site with $K_D = 0.77 \times 10^{-4}$ M has been found for manganese(II) (Mildvan and Cohn, 1963). The value of ϵ_b is temperature dependent varying between 4.5 at 4.0° and 5.9 at 35.7° (at 24.3 MHz).

This method can also be used to study competition between different metal ions for the same binding site (Mildvan and Cohn, 1965). Thus, when $\epsilon_b > 1$, introduction of diamagnetic ions competing for the same site will result in a decrease of ϵ^*. In two experiments $CaCl_2$ was introduced to a solution of albumin and Gd(III) at a 12- and a 120-fold excess over the $GdCl_3$ present. Within experimental error no decrease in ϵ^* was observed. It is concluded that, under the conditions of the experiment (pH 6.3, 25°), calcium(II) does not bind appreciably at the site for gadolinium(III) binding, *i.e.*, $K_D(Ca) > 120\,K_D(Gd)$ for this site.

Proton Relaxation Rates. Longitudinal relaxation times of water protons in $GdCl_3$ solutions in the absence and presence of cacodylate (0.05 M) were measured at three frequencies and different temperatures. The results in terms of $1/T_{1p}$ at 10^{-4} M Gd(III) are plotted against the inverse absolute temperature in Figure 4. It is seen that the pure aquo complex is more effective than the cacodylate complex in causing relaxation indicating replacement of water by cacodylate in the first coordination sphere. For the two complexes and at the three frequencies $1/T_{1p}$ monotonically decreases with increasing temperature. The values at 8.13 MHz are higher than those at 24.3 and 60.0 MHz. Note the crossing of the curves at 24.3 and 60.0 MHz.

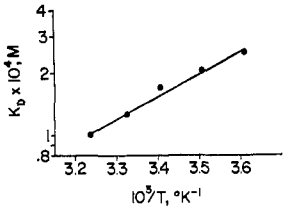

FIGURE 3: The dissociation constant of Gd(III)–albumin complex as a function of inverse absolute temperature. The slope is −5.1 kcal/mole.

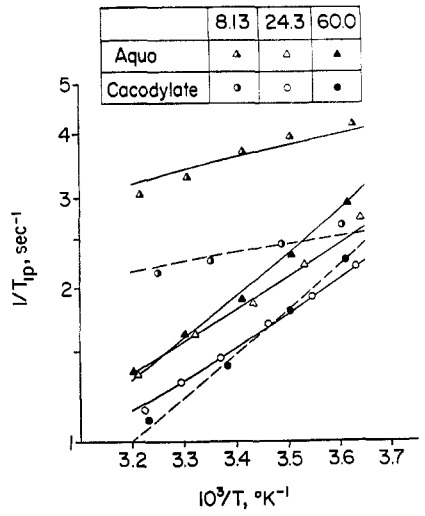

FIGURE 4: The paramagnetic contribution to the longitudinal proton relaxation rate of water due to 10^{-4} M of the aquo and cacodylate complexes of Gd(III) as a function of inverse absolute temperature at three frequencies (in MHz).

The results were analyzed in terms of the Solomon–Bloembergen equations. The term due to the electron–nuclear dipolar interaction is (*cf.* Reuben *et al.*, 1970, and references cited therein)

$$1/T_{1M} = Df(\tau_c) \tag{7}$$

$$f(\tau_c) = [3\tau_{c_1}/(1 + \omega_I^2\tau_{c_1}^2) + 7\tau_{c_2}/(1 + \omega_s^2\tau_{c_2}^2)]$$

Another term due to the hyperfine interaction is omitted since it is negligible owing to its functional form and the very low value of the hyperfine coupling constant for Gd(III) (Reuben and Fiat, 1967). In eq 7, $D = (2/15)S(S + 1)\gamma_I^2 g^2\beta^2 r^{-6}$, with S being the resultant electronic spin angular momentum (in $\hbar$ units), γ_I, the nuclear magnetogyric ratio, g, the electron g factor, β, the Bohr magneton, r, the length of the electron to nucleus vector, and ω_I and ω_s are, respectively, the nuclear and electron Larmor frequencies. The correlation times are

$$1/\tau_{c_1} = 1/\tau_r + 1/T_{1e} + 1/\tau_M \tag{8a}$$

$$1/\tau_{c_2} = 1/\tau_r + 1/T_{2e} + 1/\tau_M \tag{8b}$$

where τ_r is the characteristic time for random reorientation of $\vec{r}$ and T_{1e} and T_{2e} are, respectively, the longitudinal and transverse electron spin relaxation time. In general $T_{1e} \geq T_{2e}$. It is usually assumed that τ_r obeys a simple exponential law of the form

$$\tau_r = \tau_r^0 \exp(E_r/RT) \tag{9}$$

From results (Bernheim *et al.*, 1959) of $1/T_{1p}$ in solutions of Gd(III) and from the rates of complex formation (Geier, 1965) and water exchange (Reuben and Fiat, 1969) of the trivalent lanthanides it can be shown that the condition $T_{1M} \gg \tau_M$ holds for Gd(III). From previous results (Bernheim *et al.*, 1959) it can be shown for aquo-Gd(III) and anticipated for Gd(III)–cacodylate that $\omega_I^2\tau_{c_1}^2 \ll 1$. The same may not be true for the Gd(III)–albumin complex since τ_r as estimated from Stokes law is of the order of 10^{-8} sec.

TABLE I: Gadolinium Water–Proton Distances.

Number of waters (n)	6	7	8	9
r[aquo-Gd(III)] ± 0.10, Å	2.85	2.92	2.99	3.05
r[Gd(III)–cacodylate] ± 0.10, Å	2.98	3.06	3.13	3.19

From the ratio of the relaxation rates at two frequencies and using the values of T_{1e} and T_{2e} calculated with parameters obtained in an electron paramagnetic resonance study (J. Reuben, submitted for publication) τ_r can be evaluated. At 300°K, $\tau_r = 7.02 \times 10^{-11}$ sec. Both for aquo-Gd(III) and Gd(III)–cacodylate $\tau_r^\circ = 6.4 \times 10^{-14}$ sec and $E_r = 4.2$ kcal/mole and these values were used in calculating the curves in Figure 4. The crossing of the curves at 24.3 and 60.0 MHz is due to a larger contribution of the frequency-dependent T_{2e} to the correlation time at the lower frequency. With values for τ_r, T_{1e}, and T_{2e} the function $f(\tau_c)$ can now be evaluated at each frequency. Since $1/T_{1p}$ is proportional to n/r^6 this quantity can be calculated from the experimental results at each frequency. The average values of r for aquo-Gd(III) and Gd(III)–cacodylate obtained for several integral values of n are summarized in Table I.

From crystallographic data r can be estimated to be between 3.047 and 3.126 Å using, respectively, 2.39 and 2.42 Å for the Gd–O distance, (Marezio *et al.*, 1961) 0.95 and 1.00 Å for the O–H distance, 108 and 106° for the H–O–H angle, (Chidambaram, 1962) and assuming axial symmetry of the water molecule with respect to the Gd–O bond. For the aquo complex the average value of r obtained for $n = 9$ fits best within this range, however with the relatively large uncertainty in r, $n = 8$ is also possible. A similar conclusion has previously been reached by Morgan (1963). If one assumes that r does not change with complexation the results suggest that in the cacodylate complex of Gd(III) there are two water molecules less than in the pure aquo complex.

The enhancement of the proton relaxation rate due to the binding of gadolinium(III) to albumin was measured at the three frequencies for several protein-containing samples at a constant Gd(III) concentration of 4.21×10^{-5} M. Values of ϵ_b were calculated from eq 4 using the previously determined binding parameters (*vide supra*). From ϵ_b and using the $1/T_{1p}$ values of the cacodylate complex, at the corresponding frequency and temperature, the paramagnetic contribution to the relaxation rate due to the Gd(III)–albumin complex, $(1/T_{1p})_b$, was calculated. The results normalized to 10^{-4} M Gd(III) are graphically presented in Figure 5. Equation 7 predicts a maximum in the relaxation rate as a function of the correlation time at $\tau_{c_1} = 1/\omega_I$ and also a lower relaxation rate at the higher frequency for the same correlation time. It is seen in Figure 5 that contrary to this expectation the relaxation rates at 8.13 MHz are lower than those at 24.3 and 60.0 MHz. Similar behavior has also been observed for $(1/T_{1p})_b$ of several macromolecular complexes of manganese(II) (Reuben and Cohn, 1970). Its origin is in the frequency dependence of the correlation time, which for a macromolecular complex has a much larger contribution from the electron spin relaxation time (see eq 8) and the latter, as demonstrated by electron paramagnetic resonance is frequency dependent (J. Reuben, submitted for publication). A theoretical fit of the results for the Gd(III)–albumin complex is a difficult task. There are four unknown parameters: n, r, τ_M, and T_{1e}. Two of them (τ_M and T_{1e}) are involved functions of temperature and one (T_{1e}) is also frequency dependent. One observes, however, that $(1/T_{1p})_b$ decreases with increasing temperature at all three frequencies. This indicates that $\tau_{c_1} < 1/\omega_I$. Thus at 60.0, MHz $\omega_I = 2\pi 60 \times 10^6$ sec^{-1} and $\tau_{c_1} < 2.64 \times 10^{-9}$ sec. Using $r = 3.05$ Å a lower limit of $n = 2$ is calculated for the number of water molecules in the first coordination sphere of Gd(III) bound to the protein. An upper limit of $n = 4$ is calculated assuming $\tau_{c_1} > T_{2e}$.

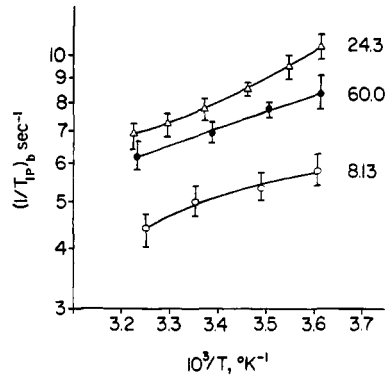

FIGURE 5: The paramagnetic contribution to the longitudinal proton relaxation rate of water due to 10^{-4} M Gd(III) bound to albumin as a function of inverse absolute temperature at three frequencies (in MHz). Lines are drawn by eye. The bars represent the scatter of values obtained for five to nine samples of different protein concentrations at a constant Gd(III) concentration.

Discussion

The paramagnetic contribution to the longitudinal proton relaxation rates of water due to gadolinium(III) are significantly enhanced in the presence of bovine serum albumin. The enhancement can be used as an analytical parameter in studying the binding of Gd(III) to biological macromolecules. The number of binding sites and the dissociation constant for the Gd(III)–albumin complex have been evaluated. The binding sites of albumin for Gd(III) seem to be different from those for calcium. A recent suggestion (Birnbaum *et al.*, 1970) that lanthanides can be used as probes of "electrostatic binding sites in proteins" remains thus unfulfilled. However, no general conclusions in this respect should be drawn since albumin is a protein of rather unusual binding properties and little selectivity.

The enhancement of the Gd(III)–albumin complex is about 5 at 24.3 MHz. Similar enhancements have been observed for Gd(III) bound to beef heart mitochondria (at 24.3 MHz, 26°, and in cacodylate buffer)[2] and lysozyme (40°, unspecified frequency and buffer) (Morallee *et al.*, 1970). Higher enhancements may be obtained with other macromolecules, *e.g.*, an enhancement of 10 has been found for Gd(III) bound to pyruvate kinase (at 24.3 MHz, 25°, and in cacodylate buffer) (J. Reuben, unpublished experiments). The enhancement results from a change in the dominant correlation time modulating the dipolar electron–nuclear interaction. For the aquo and cacodylate complexes of Gd(III) this is primarily the tumbling time of the complex, whereas for the Gd(III)–albumin complex it is to a large extent the electron spin relaxation time.

[2] Unpublished experiments done in collaboration with Drs. B. Chance and S. Fleischer.

Gadolinium(III) appears to be a suitable paramagnetic probe for studying the macromolecular environment of metal ions by magnetic relaxation methods. Other members of the lanthanides series, with very short electron spin relaxation times (Reuben and Fiat, 1969) can be used to produce isotropic nuclear resonance shifts (Morallee *et al.*, 1970).

Acknowledgments

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Study of the Nature of the Metal-Binding Sites and Estimate of the Distance between the Metal-Binding Sites in Transferrin Using Trivalent Lanthanide Ions as Fluorescent Probes*

Chun Ka Luk

ABSTRACT: Trivalent lanthanide ions were used as fluorescent probes in the study of transferrin conformation. It was found that there are two specific binding sites per transferrin molecules for Tb^{3+}, Eu^{3+}, Er^{3+}, and Ho^{3+}, and that there is only one specific binding site per transferrin molecule for Nd^{3+} and Pr^{3+}. The latter ions have larger ionic radii than the former. It was also shown that two tyrosyl residues are involved in each terbium binding site. Studies by fluorescence spectroscopy showed that terbium ion is bound to the phenolic oxygen of the tyrosyl residues. The small deuterium solvent effect on the Tb^{3+} fluorescence in the complex indicates that very few water molecules are bound to terbium.

From the lack of energy transfer between a Tb^{3+} and Fe^{3+} bound to the same protein, it is found that the distance between the two specific metal binding sites is equal to or greater than 43 Å.

Human serum transferrin is an iron-binding protein acting as an iron buffer and also as an iron carrier. Transferrin, whose molecular weight is 77,000 (Mann *et al.*, 1970), is probably a prolate ellipsoid of axial ratio 1:3 (Bezkorovainy and Rafelson, 1964). It possesses two iron-binding sites which are also capable of binding a number of other metal ions specifically and tightly. Much of the chemistry of transferrin was reviewed recently (Aisen, 1971). In summary, it has been found that the two binding sites are probably equivalent and greater than 9 Å apart; that four nitrogen ligands are available at each site; that a few tyrosyl residues are probably involved in metal binding; and that bicarbonate is involved in the iron binding.

In this paper, fluorescence techniques were used to study the nature of metal-binding sites and the separation of the two binding sites. Fluorescent probes were used and measurements on energy transfer were involved. The fluorescent probes used were trivalent lanthanide ions and ferric and cupric ions were used as quenchers.

Experimental Section

Purified human transferrin (iron free) was purchased from Behring Diagnostics, Inc. (Woodbury, N. Y.), and was used

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