# The Lanthanide Cations as Nuclear Magnetic Resonance Probes of Biological Systems

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*Summary* The lanthanide cations are shown to be excellent n.m.r. probes of their immediate co-ordinated neighbours and of their environment in enzymes including more distant molecules such as bound substrates.

Two recent publications[1,2] on the use of europium(III) as a shift reagent prompt us to publish this report on our wider use of lanthanide ions especially $Eu^{III}$, $Ho^{III}$, and $Gd^{III}$ as structural probes in biochemistry.[3]

In order to appreciate the magnitude and nature of the chemical shifts of resonance in $^1H$ n.m.r. spectra of ligands by lanthanide ions we have first made an extensive study of the effect of $Eu^{III}$, a cation of very short electron relaxation time,[4] on the spectra of EDTA, EGTA, and NTA† in $D_2O$ over a range of ligand concentrations and temperatures (−10 to 100°). The results for EDTA, the only ones to be mentioned here, together with crystallographic data,[5] permit a description of the structure of the molecule in solution in terms of its diastereoisomers. At the lowest temperatures a discrete spectrum of the particular structures present is seen whereas at the higher temperatures, above 30°, an averaged spectrum due to conformational flipping and epimerization is observed. Europium(III) is then an excellent probe of tightly bound ligands, in both structural and kinetic respects, as well as of its loosely bound ligands. Its binding site and the dynamics of its conformational equilibria in biological molecules should be readily defined using high resolution $^1H$ n.m.r.

We turn next to an examination of the site of binding of more distant molecules bound in a protein but outside the co-ordination sphere of the cation. We have chosen lysozyme as a model protein, as *X*-ray studies[6] have shown that $Gd^{III}$ (and therefore presumably all lanthanides) binds in the active site between Asp-52 and Glu-35. The following lanthanide cations were chosen for test experiments: $Eu^{III}$ as a shift probe as it is expected to have the fastest electron relaxation time,[4] $Ho^{III}$ as a shift (and possibly broadening) probe as it has the largest susceptibility,[7] and $Gd^{III}$ as a broadening probe as it is expected to have one of the longest electron relaxation times.[4,8]

Titrations of lysozyme in pD range 5—6 at 40° with $Eu^{III}$ and $Ho^{III}$ have shown specific shifts of the $^1H$ n.m.r. resonances of the enzyme corresponding to the formation of 1 : 1 complexes with dissociation constants of $1{\cdot}1 \times 10^{-2}$ mol $l^{-1}$. Association of a substrate analogue, β-methyl-*N*-acetylglucosamine (β-Me-NAG), with the $Eu^{III}$–lysozyme complex results in the formation of a 1 : 1 complex (dissociation constant of $4{\cdot}2 \pm 1 \times 10^{-2}$ mol $l^{-1}$) and causes shifts of the resonances of the acetamido and glycosidic methyl groups of +1·6 and −2·8 p.p.m., respectively. After correction for the effects[9] of the enzyme, the shifts due to the paramagnetism of the $Eu^{III}$ ion are *ca.* +1·1 and −3·0 p.p.m.

Dipolar paramagnetic shifts are given by[10]

$$\left[\frac{\Delta\nu}{\nu}\right]_i^{\mathrm{dip}} = \frac{DG_i}{r_i^3} \qquad (1)$$

where $r_i$ is a vector from the metal to the *i*th proton, $G_i$ is a function of the angle between $r_i$ and the *g* tensor of the metal complex, and *D* is a function of temperature and the magnetic properties of the complex. In previous studies of shifts caused by $Eu^{III}$,[1,2] $G_i$ was assumed to be constant and the dipolar shifts were directly related to $r_i^{-3}$. The observation of opposite signs for the above shifts indicates that this approximation cannot be made and the ratio of the $r_i$ values cannot be evaluated directly for the sugar lysozyme complex.

Association of β-Me-NAG with $Ho^{III}$–lysozyme results in both broadening and shifts of the sugar methyl resonances, particularly the resonance of the glycosidic methyl protons.

With $Gd^{III}$–lysozyme appreciable broadening of all the sugar resonances was observed before any shifts could be detected. The observation of one set of peaks for both free and complexed sugar molecules and a negligible frequency dependence (at 60 and 100 MHz) of the line broadening indicates that the exchange is fast and that

$$[T_{2p}^{-1}]_i \equiv [T_2^{-1}]_i - [T_{2o}^{-1}]_i = f[T_{2M}^{-1}]_i$$

where $[T_2^{-1}]_i$ is the observed transverse relaxation rate of the *i*th proton, $[T_{2p}^{-1}]_i$ is the contribution of the enzyme-bound metal to the observed rate, $[T_{2o}^{-1}]_i$ is the contribution of other mechanisms, $[T_{2M}^{-1}]_i$ is the relaxation rate of the fully complexed sugar protons, and *f* is the fraction of complexed sugar. $T_{2M}^{-1}$ was calculated to be $5{\cdot}3 \times 10^3$ and $17{\cdot}2 \times 10^3$ $s^{-1}$ for the acetamido and glycosidic methyl protons, respectively, at 40°.

Neglecting contact contributions $[T_{2M}^{-1}]_i$ is given by[8,11]

$$[T_{2M}^{-1}]_i = \frac{1}{15}\frac{\mu^2\gamma_H^2}{r_i^6}\left[7\tau_c + \frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right] \qquad (2)$$

where $\mu$ is the magnetic moment[7] of the gadolinium, $\omega_S$ the Larmor angular precession frequency of the electron, $\gamma_H$ the gyromagnetic ratio of the proton, and $\tau_c$ the correlation time for the magnetic perturbations. As $\mu$, $\tau_c$, and $\omega_S$ are constant for a given complex, the ratios of $r_i$ can be calculated directly from the values of $[T_{2M}^{-1}]_i$. Thus for the sugar methyl protons $r_{\mathrm{acetamido}} : r_{\mathrm{glycosidic}} = 1{\cdot}20 \pm 0{\cdot}05$.

Absolute $r_i$ values can only be calculated if $\tau_c$ is known. A pulse n.m.r. study[12] of the temperature and frequency dependence of the proton relaxation rates in aqueous solutions of $Gd^{III}$ showed that the relevant correlation time ($\tau_c = 3{\cdot}5 \pm 0{\cdot}5 \times 10^{-11}$ s at 40°) was that for rotation. Further support for this comes from the observation of an

† Ethylenediaminetetra-acetic acid, ethyleneglycolbis(aminoethyl)tetra-acetic acid, and nitrilotriacetic acid, respectively.

e.s.r. signal in these solutions indicating a probable electron spin relaxation time of *ca.* $10^{-9}$ s. These observations mean that $Gd^{III}$ can be used as a probe in proton relaxation enhancement (P.R.E.) studies (*cf.* the use of $Mn^{II}$ [11,13]). The enhancement for a fully formed $Gd^{III}$–lysozyme complex was found to be $4{\cdot}80 \pm 0{\cdot}05$ at 40°. This gives a value of $\tau_c$ for the complex of $2{\cdot}2 \pm 0{\cdot}5 \times 10^{-10}$ s assuming the number of water molecules in the first co-ordination sphere of $Gd^{III}$ is reduced from 9 to 7 on binding to the enzyme. Using this value of $\tau_c$, $r_{acetamido}$ and $r_{glycosidic}$ are calculated to be $6{\cdot}5 \pm 0{\cdot}3$ Å and $5{\cdot}4 \pm 0{\cdot}4$ Å, respectively. In evaluating $\tau_c$ no allowance was made for a possible reduction in dipolar coupling due to rapid rotations of the bound water molecules about the Gd–O($H_2O$) bonds. This reduction will not be more than 20% [11,14] giving maximum values of $\tau_c$, $r_{acetamido}$ and $r_{glycosidic}$ of $2{\cdot}7 \times 10^{-10}$ s, 6·7 Å, and 5·6 Å, respectively. A further reduction due to rotation of the $Gd(H_2O)_n$ unit about the Gd–enzyme bonds is not expected, as the *X*-ray results[6] indicate that it is held rigidly by bonds to the side chains of residues Asp-52, Glu-35, and possibly also Asn-46 and Asn-44.

Low resolution (6 Å) *X*-ray studies[15] have shown that β-Me-NAG binds to lysozyme in the same way as β-NAG.[16] Using a model of lysozyme with a β-Me-NAG molecule positioned according to the high-resolution co-ordinates of β-NAG the distances of the acetamido and glycosidic methyl protons from the binding site of the $Gd^{III}$[6] were measured. It was found that $r_{acetamido}$ is $7{\cdot}3 \pm 0{\cdot}8$ Å; however, tilting of the sugar molecule about the hydrogen bonds between the acetamido side-group and the main chain CO and NH groups of amino-acid residues 107 and 59, respectively, reduced this distance to 6·8 Å. Also, $r_{glycosidic}$ varied from 4·0—4·8 Å depending on the degree of rotation of the methoxy-group about the C-1–O bond. The most probable value was found to be 4·6 Å taking into consideration interactions with the $Gd^{III}$ and the protein side chains. Thus, the $r_i$ values determined from the *X*-ray and $^1H$ n.m.r. data are in close agreement.

These studies of lysozyme show the way in which a lanthanide probe can be used in an enzyme which does not contain a metal. Obviously a metallo-enzyme will contain a site of higher binding constant for metals and thus the method is more appropriate to such enzymes. The lanthanides match in size the calcium cation and in order to determine the full potential of the method we are studying staphylococcal nucleus (with Professor C. B. Anfinsen) using both lanthanide(III) cations and europium(II).

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