(14) K. Wuthrich, *Struct. Bonding (Berlin)*, **8**, 53 (1970).
(15) (a) G. Redfield and R. J. Gupta, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 405 (1971); (b) C. C. McDonald and W. D. Phillips, *Biochemistry*, **12**, 3170 (1973).
(16) (a) R. M. Keller, G. W. Pettigrew, and K. Wuthrich, *FEBS Lett.*, **36**, 151 (1973); (b) C. C. McDonald, W. D. Phillips, and J. LeGall, *Biochemistry*, **13**, 1952 (1974); (c) G. M. Smith and M. D. Kamen, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4303 (1974).
(17) A. Kowalsky, *Biochemistry*, **4**, 2382 (1965).
(18) H. Davenport, *Nature (London)*, **169**, 75 (1952).
(19) F. Sherman, J. W. Stewart, J. H. Parker, E. Inhaber, N. A. Shipman, G. J. Putterman, R. L. Gardisky, and E. Margollash, *J. Biol. Chem.*, **243**, 5446 (1968).
(20) H. Muir and A. Neuberger, *Biochem. J.*, **45**, 163 (1949).
(21) H. Budzikiewicz, C. Djerassi, and D. Williams, "Mass Sepctrometry of Organic Compounds", Holden-Day, San Francisco, Calif., 1967, pp 628–629.
(22) A. N. H. Yeo, *Chem. Commun.*, 1154 (1970).
(23) T. Popper and H. Tuppy, *Acta Chem. Scand.*, **17**, S47 (1963).
(24) (a) H. Theorell, *Biochem. Z.*, **301**, 201 (1939); (b) D. Theodoropoulos and I. Souchleris, *J. Org. Chem.*, **31**, 4009 (1966); (c) H. Gnichtel and W. Lautsch., *Chem. Ber.*, **98**, 1647 (1965).
(25) (a) H. Theorell, *Biochem. Z.*, **295**, 207 (1936); (b) *ibid.*, **298**, 242 (1938); (c) *ibid.*, **301**, 201 (1939); (d) *Enzymologia*, **4**, 192 (1937).
(26) J. B. Paine and D. Dolphin, *J. Am. Chem. Soc.*, **93**, 4080 (1971).
(27) R. Bonnett, I. A. D. Gale, and G. F. Stephenson, *J. Chem. Soc. C*, 1600 (1966).
(28) S. J. Jacobson, C. G. Willson, and H. Rapoport, *J. Org. Chem.*, **39**, 893 (1974).
(29) G. W. Kenner, S. W. McCombie, and K. M. Smith, *Justus Liebigs Ann. Chem.*, 1362 (1973).
(30) R. P. Carr, A. H. Jackson, G. W. Kenner, and G. S. Sach, *J. Chem. Soc. C*, 487 (1971).
(31) The small but distinct differences between synthetic and natural porphyrin c will be discussed in a future publication.
(32) Solvent evaporations were carried out in vacuo using a Berkeley rotary evaporator, ultraviolet and visible spectra were measured on a Cary Model 14 spectrometer, NMR spectra were taken on Varian Model T-60, HA-100, and HR-220 spectrometers using internal Me_4Si, and mass spectra were obtained on CEC 103 and 110B and A.E.I. MS9 spectrometers. The 200-tube automatic Craig type distribution train was manufactured by H. O. Post Scientific Instrument Co. Radioactivity determinations were made with Packard TriCarb equipment; sample oxidation in sample oxidizer, Model 305, and counting in liquid scintillation counter, Model 3375. Elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley.
(33) V. du Vigneaud, L. F. Audreith, and H. S. Loring, *J. Am. Chem. Soc.*, **52**, 4500 (1930).
(34) D. Seebach, B. Erickson, and G. Singh, *J. Org. Chem.*, **31**, 4303 (1966); A. Vallet, A. Janin, and R. Romanet, *J. Labelled Compd.*, **4**, 299 (1968).
(35) C. S. Marvel and F. Hager, "Organic Syntheses", Collect. Vol. I, Wiley, New York, N.Y., 1944, p 248.
(36) The preparation of $[2\text{-}^2H_1]$ethanol has been reported via $LiAlD_4$ reduction of ethylene oxide by E. Bengsch and M. Corval, *Bull. Soc. Chim. Fr.*, 1867 (1963). In our hands this procedure resulted in significant quantities of both dideuterio and unlabeled products. The isotopic scramble is difficult to detect by NMR or by mass spectral analysis of the alcohol because pressure-dependent intermolecular proton transfer in the mass spectrometer and the natural isotopic mixture of oxygen renders the M^+ region complex. In the derived iodide, these problems are both obviated and the molecular ion region of the iodide mass spectrum is uncomplicated and accurately reveals this isotopic mixture.
(37) M. M. Kreevoy and M. A. Turner, *J. Org. Chem.*, **30**, 373 (1965).

Lanthanide Interactions with Nitrotyrosine. A Specific Binding Site for Nuclear Magnetic Resonance Shift Probes in Proteins

Timothy D. Marinetti, Grayson H. Snyder, and Brian D. Sykes*

Contribution from the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received March 20, 1975*

Abstract: A 100-MHz ^1H NMR study of the relaxation and chemical shifts induced by the interaction of Eu(III), Pr(III), Gd(III), and La(III) with *N*-acetyl-L-3-nitrotyrosine ethyl ester has been undertaken to characterize the nitrotyrosine residue as a potential specific lanthanide metal binding site in proteins. The NMR data, together with independent measurements of the metal–ligand stability constants, give the parameters of the dipolar interaction. These indicate a significant contribution from nonaxially symmetric terms. In addition, a nonnegligible contact shift is present for Eu(III).

The utility of the lanthanide shift probes as an aid in determining molecular structure and conformation in solution has been well documented over the last several years.[1,2] Most of the work to date has involved the use of "shift reagents" [e.g., $Ln(fod)_3$] in organic solvents. Increasingly more interest is being shown in using the aquo cations themselves, or their EDTA complexes as aqueous shift reagents, for structure determination in aqueous solution. In particular, several molecules of biochemical interest have been studied including mononucleotides,[3,4] dinucleotides,[5] and membranes.[6,7]

Recently the Oxford enzyme group has been investigating the use of the lanthanide binding to a specific site on the protein lysozyme to determine the three-dimensional structure of the protein in solution with minimal reference to the known X-ray structure.[8,9] A limitation of the extension of this technique to other proteins is that the protein must possess a single specific naturally occurring binding site. Although the lanthanides are reported to bind to several proteins such as thermolysin,[10] α-amylases,[11] trypsinogen,[12] and ferredoxin,[13] this will obviously not be true for all proteins of interest. One way to circumvent this difficulty is the incorporation of a binding site into the protein.[9] We have therefore investigated the chelating group nitrotyrosine which can be introduced with relative ease into a large number of proteins.[14] In many of these proteins, nitration occurs at only one tyrosine, hence generating a single binding site for the metal ion.

This paper reports the study of the interaction of Pr(III), Eu(III), and Gd(III) with the model peptide *N*-acetyl-L-3-nitrotyrosine ethyl ester by measurement of the induced NMR shifts, line widths, and spin-lattice relaxation times. In addition, by potentiometric titration, the binding constants of these metals and La(III), a diamagnetic analog of the 4f series, have been determined. With known binding constants, the equilibrium composition of the samples used in NMR can be calculated, and, hence, data involving a change in a spectral parameter as a function of the concentration of paramagnetic metal ion can be analyzed to extract the parameters for the metal–ligand complex. Using the isotropic relaxation probe Gd(III) and the known X-ray structure of nitrophenol, the location of the metal binding

---

* Address correspondence to this author at Department of Biochemistry, The University of Alberta, Edmonton, Alberta, Canada T6G-2H7.

site and the atomic coordinates in the metal complex were calculated. Since the nitrotyrosine ring is rigid, the ring protons provide a test of the validity of the dipolar model in predicting lanthanide-induced shifts. Our analysis of the NMR shifts indicates that the dipolar model works very well for Pr(III) with the contact contribution estimated to be less than 2% of the smallest shift. For Eu(III), the results indicate that a significant contact interaction is involved, but the dipolar contribution is still dominant. Operational parameters have been obtained for use of the nitrotyrosine residue as a chelating site in proteins. Using these parameters, the observed lanthanide-induced shifts in the NMR spectrum of a nitrated protein can give information about the placement of the observed residue relative to the metal. Finally, a general theoretical discussion shows that the principal axes of the dipolar interaction cannot be unambiguously determined from solution NMR measurements. Further, axial symmetry will be indicated by internal shift ratios only if the ratios are constant to within a few percent. Deviations greater than this imply that nonaxial terms are contributing to the shifts.

Theory. Following Bleaney[15] and Horrocks,[16] we treat the lanthanide dipolar shifts as arising from anisotropy in the magnetic susceptibility tensor, $\boldsymbol{X}$, which is the population average of the magnetic interactions of the 4f electrons in the thermally occupied J sublevel of the ground term. When electron spin relaxation is fast compared to nuclear relaxation, the electronic magnetic moment seen by the nucleus is averaged over all thermally accessible electronic states. We consider a ligand molecule in some molecular frame, which can be related to the lab frame by a coordinate rotation $\boldsymbol{R}$. Assuming the lanthanide-induced shift is small, the nuclear spins will be quantized along the main field $\mathbf{H}$. In the molecule fixed frame the magnetic moment of the nucleus becomes $\boldsymbol{R}^{-1}\cdot\mathbf{I}$, and the field is similarly $\boldsymbol{R}^{-1}\cdot\mathbf{H}$.

The magnetic moment of the metal ion in the molecular frame is $\boldsymbol{\mu}_e = \boldsymbol{X}\cdot\boldsymbol{R}^{-1}\cdot\mathbf{H}$. The interaction energy is the magnetic interaction which, for a nuclear dipole at r in the molecular frame, can be written as

$$\mathcal{H}_D = \boldsymbol{\mu}_1\cdot\boldsymbol{\mu}_2 r^{-3} - 3(\boldsymbol{\mu}_1\cdot\mathrm{r})(\boldsymbol{\mu}_2\cdot\mathrm{r})r^{-5} \quad (1)$$

with $\boldsymbol{\mu}_1 = \gamma\hbar\boldsymbol{R}^{-1}\cdot\mathbf{I}$, $\boldsymbol{\mu}_2 = \boldsymbol{\mu}_e = \boldsymbol{X}\cdot\boldsymbol{R}^{-1}\cdot\mathbf{H}$. This can be rewritten in tensor notations: $\mathcal{H}_D = \boldsymbol{\mu}_1\cdot\boldsymbol{D}\cdot\boldsymbol{\mu}_2$ where $\boldsymbol{D}$ is the dipole–dipole interaction tensor.[17] Substituting for $\boldsymbol{\mu}_1$ and $\boldsymbol{\mu}_2$

$$\mathcal{H}_D = (\gamma\hbar\boldsymbol{R}^{-1}\cdot\mathbf{I})\cdot\boldsymbol{D}\cdot\boldsymbol{X}\cdot\boldsymbol{R}^{-1}\cdot\mathbf{H} = \gamma\hbar\mathbf{I}\cdot\boldsymbol{R}\cdot\boldsymbol{D}\cdot\boldsymbol{X}\cdot\boldsymbol{R}^{-1}\cdot\mathbf{H} \equiv \gamma\hbar\mathbf{I}\cdot\boldsymbol{T}\cdot\mathbf{H} \quad (2)$$

where $\boldsymbol{T} = \boldsymbol{R}\cdot\boldsymbol{D}\cdot\boldsymbol{X}\cdot\boldsymbol{R}^{-1}$. This is formally analogous to a chemical shielding tensor, as can be seen by writing out the complete Hamiltonian for the nuclear spin I

$$\mathcal{H} = -\gamma\hbar\mathbf{I}\cdot(\mathbf{1} - \boldsymbol{\sigma})\cdot\mathbf{H} + \gamma\hbar\mathbf{I}\cdot\boldsymbol{T}\cdot\mathbf{H} \quad (3)$$

where $\boldsymbol{\sigma} = \boldsymbol{R}\cdot\boldsymbol{\sigma}'\cdot\boldsymbol{R}^{-1}$ and $\boldsymbol{\sigma}'$ is the screening tensor in the molecule fixed frame.

Averaging over all possible molecular orientations replaces the tensor quantities by their scalar average $(1/3)\mathrm{tr}\{\boldsymbol{T}\}$ and hence the effect of the metal ion is to shift the nuclear resonance by an amount $(-1/3)\mathrm{tr}\{\boldsymbol{T}\}$. By analogy with the screening tensor this is dimensionless and represents the relative shift $\Delta\omega/\omega_0$. Since the trace is invariant under similarity transform, such as spatial rotation, we are free to choose our coordinate system. Taking it as the principal axis system (in which $\boldsymbol{X}$ is diagonal)

$$(\Delta\omega/\omega_0) = -(1/3)\mathrm{tr}\{\boldsymbol{D}\cdot\boldsymbol{X}\} = -(1/3)(D_{xx}X_{xx} + D_{yy}X_{yy} + D_{zz}X_{zz}) \quad (4)$$

From the explicit forms of the dipole tensor, and using the fact that $\mathrm{tr}\{\boldsymbol{D}\} = 0$, the above expression reduces to

$$(\Delta\omega/\omega_0) = \tfrac{1}{2}r^{-3}[(X_{xx} - \bar{X})(3\cos^2\theta' - 1) + (X_{xx} - X_{yy})(\sin^2\theta'\cos 2\phi')] \quad (5)$$

with $\bar{X} = (1/3)\mathrm{tr}\{\boldsymbol{X}\}$, and the polar coordinates of the nucleus (r,θ',ϕ') are in the principal axis system. The above is identical with Bleaney's result,[15] except for the factor of N, which is absorbed into our $\boldsymbol{X}$ tensor. Using normalized spherical harmonics[18]

$$\tfrac{1}{2}r^{-3}(3\cos^2\theta' - 1) = r^{-3}C_{20}(\theta',\phi')$$

$$\tfrac{1}{2}r^{-3}(\sin^2\theta'\cos 2\phi') = \sqrt{1/6}\,r^{-3}(C_{22}(\theta',\phi') + C_{2-2}(\theta',\phi')) \quad (6)$$

the shift is

$$(\Delta\omega/\omega_0) = r^{-3}[(X_{zz} - \bar{X})C_{20}(\theta',\phi') + \sqrt{1/6}(X_{xx} - X_{yy})(C_{22}(\theta',\phi') + C_{2-2}(\theta',\phi'))] \quad (7)$$

If we start in some arbitrary coordinate system, in which we know the coordinates (r,θ,ϕ) of all atoms of interest and wish to know what the shift is, the procedure involves calculating the geometrical functions and seeing how well the data are fit to a simple linear combination of them. Let the real principal axis be at Euler angles $\Omega \equiv \lambda, \mu, \nu$ from the initial coordinate system. To calculate $C_{20}(\theta',\phi')$, $C_{22}(\theta',\phi')$, and $C_{2-2}(\theta',\phi')$ in the principal axis, we calculate values (in the starting system) of the $C_{2m}(\theta,\phi)$ and note that the transformation properties of the $C_{kn}(\theta,\phi)$ under rotation are well characterized[18]

$$C_{kn}(\theta',\phi') = \sum_m D_{mn}{}^k(\Omega)C_{km}(\theta,\phi) \quad (8)$$

where (θ,ϕ) are the coordinates of the point in the arbitrary coordinate system and $D_{mn}{}^k(\Omega)$ is a Wigner rotation matrix element. Using this, and the fact that r is invariant under rotation, the shift becomes

$$(\Delta\omega/\omega_0) = r^{-3}\sum_m [(X_{zz} - \bar{X})D_{m0}{}^2(\Omega) + \sqrt{1/6}(X_{xx} - X_{yy})(D_{m2}{}^2(\Omega) + D_{m-2}{}^2(\Omega))]C_{2m}(\theta,\phi) \quad (9)$$

Not knowing the principal axis transform angles λ, μ, ν, we select arbitrary angles α, β, γ and *fit* the data to a function of the form

$$(\Delta\omega/\omega_0) = r^{-3}\sum_m [A_1D_{m0}{}^2(\alpha,\beta,\gamma) + A_2(D_{m2}{}^2(\alpha,\beta,\gamma) + D_{m-2}{}^2(\alpha,\beta,\gamma))]C_{2m}(\theta,\phi) \quad (10)$$

the success of which will be determined by how well the dipolar interaction can account for the observed shifts. Since the parameters A_1, A_2, α, β, and γ must be the same regardless of r, θ, ϕ, i.e., for any nucleus in the paramagnetic complex, then each of the coefficients must separately be equal

$$A_1D_{m0}{}^2(\alpha,\beta,\gamma) + A_2(D_{m2}{}^2(\alpha,\beta,\gamma) + D_{m-2}{}^2(\alpha,\beta,\gamma)) = (X_{zz} - \bar{X})D_{m0}{}^2(\lambda,\mu,\nu) + \sqrt{1/6}(X_{xx} - X_{yy})(D_{m2}{}^2(\lambda,\mu,\nu) + D_{m-2}{}^2(\lambda,\mu,\nu)) \quad (11)$$

for $m = 0, \pm1, \pm2$. We have, in effect, six unknowns (λ, μ, ν and the principal values of the susceptibility) and a system of five equations relating them to quantities A_1, A_2, α, β, and γ which are determined by a fit to the observed chemical shifts. The situation is therefore underdetermined and there are an infinite number of solutions satisfying eq 11.

One of these solutions will have $A_2 = 0$, which represents a "pseudo axial" solution to the NMR shifts. Therefore, fitting the NMR data to an axial model is not significant in itself, since it will be possible to fit any dipolar shift with an axial expression by choice of A_1, α, β, and γ. We must ask if the resulting axes are physically reasonable or, to turn the question around, if a set of physically reasonable axes can produce values of A_1 and A_2 where $A_2 \ll A_1$; then we would be justified in speaking of an axially symmetric system.

Let the values of a α, β, and γ which give agreement with the data and yield $A_2 = 0$ be denoted α_1, β_1, and γ_1. (It can be shown that γ may be arbitrarily specified: $A_2 = 0$ eliminates the only term which contains a γ dependence.) Going back to the eq 11, we note that regarding the pseudo-axial parameters as fixed and the $\boldsymbol{X}$ and λ, μ, ν parameters as variable, we again have a situation of five equations and six unknowns. Therefore it is impossible to determine a *unique* $\boldsymbol{X}$ and λ, μ, ν which will be consistent with the pseudo-axial parameters.

One commonly used criterion of axial symmetry is that the ratio of induced shifts for any pair of nuclei is constant regardless of the lanthanide metal used.[3] If we calculate the shift in the pseudo-axial representation, then each shift is a single sum of terms involving $D_{m0}^2(\alpha_1,\beta_1,\gamma_1)$, Equating the ratio of shifts for nuclei *i* and *j* for metals M and N, and cross multiplying the two fractions yields the following

$$\sum_m \sum_p D_{m0}^2(\zeta^{\mathrm{M}}) D_{p0}^2(\zeta^{\mathrm{N}}) C_{2m}(\Phi_i) C_{2p}(\Phi_j) = \sum_m \sum_p D_{m0}^2(\zeta^{\mathrm{N}}) D_{p0}^2(\zeta^{\mathrm{M}}) C_{2m}(\Phi_i) C_{2p}(\Phi_j) \quad (12)$$

where $\zeta^{\mathrm{L}} \equiv \alpha_1^{\mathrm{L}}, \beta_1^{\mathrm{L}}, \gamma_1^{\mathrm{L}}$ (L = M, N) and $\Phi_r \equiv (\theta_r,\phi_r)$ ($r = i, j$). To be true for all nuclei *i* and *j* this requires

$$D_{m0}^2(\zeta^{\mathrm{M}}) D_{p0}^2(\zeta^{\mathrm{N}}) = D_{m0}^2(\zeta^{\mathrm{N}}) D_{p0}^2(\zeta^{\mathrm{M}}) \quad (13)$$

which in general requires $\zeta^{\mathrm{M}} = \zeta^{\mathrm{N}}$, i.e., the pseudo-axial transforms must be identical if the two metals M and N are to give constant shift ratios, which requires the right-hand side of eq 11 to scale independent of *m* and the proton position. When ratios are taken, the scaling cancels. If we assume isomorphous replacement, then the polar coordinates (r,θ,ϕ) of proton *i* and the principal axis transform $\boldsymbol{D}^2(\lambda,\mu,\nu)$ are all independent of the metal in the complex. These restrictions require both $(X_{xx} - X_{yy})$ and $(X_{zz} - \bar{X})$ to scale identically if constant ratios are to be observed. As has been pointed out in discussion[3] and by measurements of crystal susceptibilities for $\mathrm{Ln(dpm)_3(4\text{-}picoline)_2}$,[19] the two susceptibility anisotropies do not scale in the same way from metal to metal. Hence, the only way for constant shift ratios to occur would be for the nonaxial term to vanish. We point out that the substitution of one metal for another need *not* be isomorphous; either the metal-ligand bond lengths or the arrangement of ligands around the metal ion or both may change as one proceeds across the 4f series. Pressure jump studies on the aquocations indicate a change in coordination number halfway through the series.[20] In this case changes in λ, μ, and ν could alter the $D_{mn}^2(\lambda,\mu,\nu)$ factors to give apparent linear scaling of both the principal anisotropies.

Another potential problem arises for small molecules in which most protons will be found in a cone where $\theta \leq 40°$. For this case the $C_{20}(\theta,\phi)$ functions dominate the $C_{2\pm2}(\theta,\phi)$ functions, so that any axially asymmetric susceptibility term is suppressed by the geometrical factors, and hence the presence of a nonzero $(X_{xx} - X_{yy})$ will cause a small variation in the observed shift ratios. Numerical estimates, confining two protons to a region of space $0 \leq \theta \leq 40°$ and $|\phi_i - \phi_j| < 40°$ show that if the $(X_{zz} - \bar{X})$ terms scaled as a ratio α, then allowing the $(X_{xx} - X_{yy})$ terms to scale by 1.5α introduces only a 15% deviation when the two principal anisotropies are about *equal*. This fortuitous independence of the shift ratios on the scaling of the principal anisotropies could therefore allow genuine deviations in shift ratio to be ascribed to experimental error. For large θ values, whenever the nonaxial term is comparable, large deviations in shift ratio would be seen. Finally, if the nonaxial term is large compared to the axial, the deviations in shift ratio from metal to metal will be dampened, since, again, one of the terms dominates the other. Thus, constant shift ratios will really only indicate axial symmetry if very severe limits are placed on what is considered constant.

In all of the above discussion, the functions $C_{2m}(\theta,\phi)$ must be averaged over any internal motions present. Such motion can lead to great simplification, as has been shown by Briggs et al.[21] for the case of a monodentate ligand with an *n*-fold rotation axis. For equal rotamer populations and $n \geq 3$, the result is to give effective axial symmetry; the dipolar interaction will reduce to a single term of form $Kr^{-3}(3\cos^2\theta - 1)$ where θ is now the polar angle from the rotation axis. In most cases the rotation axis would be the lanthanide donor bond, and, hence, regardless of metal ion, the same effective axis system would result, and strictly constant shift ratios would be observed. For $n < 3$, the interaction will be asymmetric about this axis.[22] Both results follow from the properties of the $C_{2m}(\theta,\phi)$ functions where the polar axis is taken as the lanthanide donor bond. The situation is more complex for multidentate ligands, since in this case different stereochemical arrangements of the ligand in the coordination sphere will alter the charge distribution of the coordination sphere, and the susceptibility tensor itself will be altered. Thus, averaging over conformer coordinates to calculate the average values of the $C_{2m}(\theta,\phi)$ functions must also include the effects of averaging on the susceptibility tensor.

Experimental Section

N-Acetyl-L-3-nitrotyrosine ethyl ester (ANTE) was obtained from Sigma Chemical Co., "Specpure" lanthanide metal oxides from Johnson Matthey, Ltd., D_2O from BioRad, and DCl and NaOD from Stohler Isotope Chemicals. All were used without further purification.

Metal solutions were prepared by drying the oxides at 90° for 12-24 hr, dissolving an accurately weighed amount in HCl, and then drying under aspirator vacuum with gentle heating to form the chlorides. These were cycled into D_2O by dissolving and evaporating off solvent as before three times to remove any H_2O in the metal salts. Stock solutions were made 0.4 *M* at pH <3 to prevent precipitation of the hydroxides.

ANTE solutions were prepared gravimetrically and adjusted to pH (uncorrected meter reading) with NaOD or DCl. Metal-ANTE solutions were prepared by mixing a stock ANTE solution with aliquots of the appropriate metal chloride solution. NMR solutions were 9.5 m*M* ANTE, 14.3 m*M* total lanthanide, and 0.1 *M* KCl in D_2O and contained DSS (0.2-1 m*M*) and in some cases $\mathrm{(CH_3)_4NBr}$ as internal references. Potentiometric titrations were done in 3.25 ml of 0.1 *M* KCl in D_2O containing 0.053 mmol of metal and/or 0.0165 mmol of ANTE using the method of Irving and Rossotti.[23] Aliquots of 5 or 10 µl of 0.4613 *M* NaOD in D_2O were added and the pH was recorded, up to the point of precipitation of $\mathrm{Ln(OH)_3}$ (pH ≤8.5). No attempt was made to exclude CO_2. In all cases, freshly prepared ANTE solutions were used, since the ethyl ester hydrolyzes significantly over a period of several days, as evidenced by the appearance of ethanol peaks in the NMR spectrum. Potentiometric measurements were done on a Sargent Welch Model PBL pH meter at ambient temperature 22-25°.

100-MHz NMR spectra were recorded at 25° on a Varian XL-100-15 spectrometer in Fourier transform mode locked on D_2O. T_1 data were obtained using a standard 180°-τ-90° pulse sequence.

Table I. Binding Constants and Chemical Shifts

	$L^{b,f}$	$HL^{b,f}$	$LaL^{b,e}$	GdL	PrL^{d}	EuL^{d}
Log $K_1{}^{a}$		7.09	2.29	2.51	2.61	2.65
H(2)	770.8	798.6	791.1	*c*	4.00	−1.31
H(5)	672.9	714.2	701.5	*c*	17.05	−7.42
H(6)	720.2	754.0	748.7	*c*	6.88	−3.97
α					2.56	−1.25
β,β'					2.98	−1.38
Ethyl CH_2	416.0	418.7			1.00	−0.40
Ethyl CH_3	117.7	120.8			0.87	−0.35
N-Acetyl	197.4	195.3			0.86	−0.35

[a] First stepwise association constant ±0.1 (0.1 *M* KCl in D_2O). [b] Shifts in hertz downfield from internal DSS. [c] Maximum magnitude estimate 3.4 ppm. See text. [d] In parts per million from LaL (±5%). An upfield shift is negative. [e] From analysis of Pr and Eu titrations (±5 Hz). [f] ±0.3 Hz.

Table II. Spin-Lattice Relaxation Data

	$1/T_1{}'^{a}$	$1/(T_{1(\mathrm{B})} + \tau_M)^{b}$	$(\langle r^6\rangle)^{1/6\,c}$	$r_{\mathrm{model}}{}^{d}$	$1/T_{1(\mathrm{free})}{}^{e}$
H(2)	3,782	3,623	6.01	5.97	0.38
H(5)	10,993	10,840	4.91	4.91	0.4
H(6)	2,146	1,987	6.66	6.80	0.75
Ethyl CH_2	424	265	9.02		0.71
Ethyl CH_3	490	331	9.36		0.47
N-Acetyl	549	390	8.77		1.0
DSS	159	0			0.28

[a] Observed slope of $1/T_1$ vs. fraction bound in $\sec^{-1}$. [b] $1/T_1{}' - 1/T_{1(\mathrm{DSS})}$ in $\sec^{-1}$. [c] Calculated values using eq 14 and 15 with $\tau_M = (1.4 \pm 0.8) \times 10^{-5}$ sec and $c = (5.58 \pm 0.53) \times 10^{-9}$ sec Å^{-6}. See text. [d] Using oxygen–metal bond lengths of 2.4 Å. [e] $1/T_1$ in absence of Gd(III).

Data were analyzed using a Nova 1220 computer with programs coded in BASIC.

Results

The results of a potentiometric determination of the metal-ANTE stability constants are in Table I.[24] The method is that of Irving and Rossotti[23] in which the metal-ligand stability constant is determined by analyzing potentiometric titration data for metal alone, ligand alone, and metal plus ligand. Titrations were done with metal in excess over ligand to prevent formation of species with more than one ligand per metal ion. Due to insolubility of the lanthanide metals in alkaline solution,[25] no titration could be taken further than pH 8.5. Nonlinear least-squares techniques were used to fit the titration data. Account was taken of hydrolysis of the metal, as well as a correction for the binding of Cl^- ion ($\log K_1 \leq 0.4$).[25] A value of $\log K_1 = 2.2$ was reported for La(III) binding to nitrophenol,[26] in good agreement with our value of 2.29. No correction was made for the deuterium isotope effect in the present study, but since the NMR work was done in D_2O, the measured constants will be operationally useful. They were used throughout to calculate equilibrium concentrations of free and bound ligand species in the samples used for NMR.

The first two columns of Table I also give the NMR chemical shifts of the ANTE in the deprotonated and protonated forms. These were obtained from a pH titration of an ANTE solution. The fitted pK_a was within 0.01 pH units of that determined potentiometrically, and all fitted resonances gave the same pK_a. This value also agrees with the literature value of 7.06.[26]

The α and β,β' regions were examined using water eliminated Fourier transform (WEFT)[27] to minimize the interfering HDO peak. The observed α,β,β' ABX multiplet structure changes a great deal with pH due to a difference in the titration shifts of the two inequivalent protons. The spectrum of ANTE plus La(III) at pH 6.28 is shown in Figure 2A.

Table II lists the results of the Gd(III) experiments. Using a constant total La(III) + Gd(III), the proportion of paramagnetic metal was increased and NMR shifts and T_1's were measured for ring, *N*-acetyl, and ethyl group protons. No shifts were observed, and using 0.5 Hz as the limit of detectability, the maximum estimates for the Gd-induced shift are <3.4 ppm. The T_1's are dominated by the dipolar term and were analyzed using the expression of Luz and Meiboom[28] with an additional term for outer sphere relaxation.

$$\frac{1}{T_{1\,(\mathrm{obsd})}} = \frac{P_{\mathrm{free}}}{T_{1\,(\mathrm{free})}} + \frac{P_{\mathrm{B}}}{T_{1\,(\mathrm{B})} + \tau_{\mathrm{M}}} + \frac{P_{\mathrm{B}}}{T_{1\,(\mathrm{out.})}} \qquad (14)$$

This system is actually a four-site exchange—L, HL, LaL, and GdL—but for purposes here, L, HL, and LaL are all equivalent and are lumped together as "free". Addition of La(III) to an ANTE solution up to 35% bound did not increase the $1/T_1$ of H(2) or H(6) and had a very slight effect on the $1/T_1$ of H(5). (See Figure 1 for notation.) This can be accounted for by a small contamination of Gd in the La_2O_3 ($\lesssim$30 ppm) or an increase in the correlation time which dominates H(5) relaxation. It is worth pointing out that La_2O_3 obtained from sources other than Johnson Matthey with a purity of 99.9% caused appreciable increases in the $1/T_1$ values of *all* the ring protons, indicating a significant paramagnetic contamination.

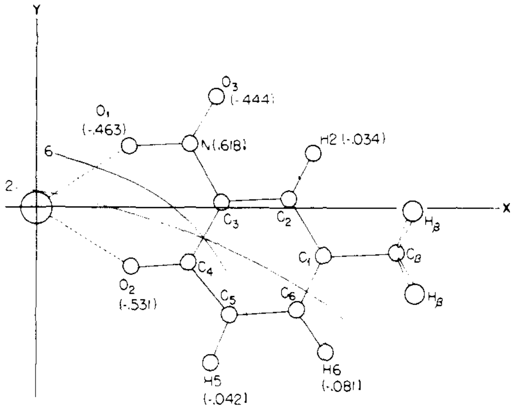

Figure 1. Scale drawing of the ring and C_β of ANTE. The metal ion defines the origin of a coordinate system in which the ANTE ring is in the *XY* plane. The metal–oxygen bond lengths are 2.4 Å. The numbers in parentheses are the electric charges computed for the indicated atoms by an INDO-AFAOS calculation on the nitrophenol anion. The lines labeled 2 and 6 are the distance restriction lines calculated from the Gd(III) T_1 data.

Outer sphere relaxation should be proportional to the number of paramagnetic ions in solution and, hence, proportional to P_{B} at low fraction bound. It was corrected for by subtracting the increase in relaxation rate of DSS from the observed slopes of a plot of $1/T_{1\,(\mathrm{obsd})}$ vs. P_{B}. These corrected relaxation rates are shown in column 2 of Table II. These are assumed equal to $1/(T_{1\,(\mathrm{B})} + \tau_{\mathrm{M}})$. To proceed to get geometry, the contribution of τ_{M} must be evaluated. $T_{1\,(\mathrm{B})}$ is given by[29]

$$\frac{1}{T_{1\,(\mathrm{B})}} = \frac{2}{15}\,\frac{\gamma_{\mathrm{I}}^2 g^2 \beta^2 J(J+1)}{r^6} \times \left(\frac{3\tau_{\mathrm{c}}}{1 + \omega_{\mathrm{I}}^2\tau_{\mathrm{c}}^2} + \frac{7\tau_{\mathrm{c}}}{1 + \omega_{\mathrm{S}}^2\tau_{\mathrm{c}}^2}\right) \equiv \frac{1}{cr^6} \qquad (15)$$

There are more variables than data, so the distances τ_{M} and *c* cannot be uniquely determined. However, a lower bound to the distance ratios can be obtained by comparing the relaxation rates. The ratio of relaxation rates for protons *i* and *j* is given by

$$\frac{(1/T_1)_i}{(1/T_1)_j} = \frac{cr_j^6 + \tau_M}{cr_i^6 + \tau_M} \le \left(\frac{r_j}{r_i}\right)^6$$

or $\qquad\qquad$ (16)

$$\frac{r_j}{r_i} \ge \left(\frac{(1/T_1)_i}{(1/T_1)_j}\right)^{1/6}$$

for i and j chosen to make $(1/T_1)_i \ge (1/T_1)_j$. Using the data of column 2 in Table II, $r_2/r_5 \ge 1.20$ and $r_6/r_5 \ge 1.33$. Figure 1 shows a drawing of the ANTE ring and C_β with the two curves corresponding to $r_2 = 1.20r_5$ and $r_6 = 1.33r_5$ drawn in. The inequalities confine the metal to the region closest to H(5). Thus the metal must be binding to the phenol alone or to the nitro and phenol oxygens as a bidentate ligand. A metal-oxygen distance of 2.4 Å was estimated from X-ray data for the lanthanide ethyl sulfates,[30] hydroxyacetates,[31,32] and oxalates.[33] Using this distance, the monodentate case places the metal too close to H(5) relative to H(2) and H(6). The relaxation rate of H(5) would have to be much larger relative to H(2) and H(6) than is observed. Using bidentate coordination, proton-metal distances are calculated from the known structure of nitrophenol.[34] These are shown in column 4 of Table II. The corrected $1/T_1$ slopes are now fit to eq 14 and 15 and values of $c = 5.58 \times 10^{-9}$ sec Å^{-6} and $\tau_M = 1.4 \times 10^{-5}$ sec are obtained. The distances calculated with these parameters are given in column 3, and as can be seen, the calculation is self-consistent. An independent check can be obtained from pressure jump data[20] for the forward step of ligation for oxalate and murexide[35] binding to lanthanide metals. Both are bidentate oxygen ligands and should behave similar to ANTE. The authors report forward rate constants which are independent of ligand, presumably because removal of water from the primary coordination sphere is the rate-limiting step. The observed k_1 value starts at $\sim 8 \times 10^7$ sec^{-1} M^{-1} for the light lanthanides, drops to 4×10^7 at Gd, and falls quickly afterward, eventually reaching 0.6×10^7 for Yb. Using our measured stability constant, we can calculate a τ_M from the pressure jump k_1

$$\tau_M = 1/k_{-1} = K_1/k_1 = 10^{2.51}/4 \times 10^7 = 8 \times 10^{-6}\ \text{sec} \tag{17}$$

which is in reasonable agreement with our value of 1.4×10^{-5} sec.

From the known ω_I and ω_s, we may calculate τ_c from eq 15 using the fitted value for c. There are two solutions due to the quadratic dependence on τ_c, corresponding to $\tau_c = 2.2 \times 10^{-8}$ and 1.2×10^{-10} sec. The second figure is close to the rotational correlation time expected for a molecule the size of the Gd-ANTE complex. It may be compared with a reported τ_c for the $Gd(H_2O)_9^{3+}$ ion of 4×10^{-11} sec[36] which is known to be dominated by τ_{rot}. If the Gd-ANTE τ_c is τ_{rot} it implies that the electron spin relaxation time must be longer than 10^{-10} sec.

Our data are therefore consistent with metal binding at the bidentate site formed by the nitro and phenol oxygens. Further, the excellent agreement of the T_1 data with a dipolar model shows that the scalar A **I**·**J** interaction is small. This is supported by the fact that no Gd-induced shifts were observed.

Relaxation data were also measured for Pr and Eu, but the increases in $1/T_1$ were very small: unobservable for Eu and $1/T_{1(B)}$ of 6.3, 18, and 4.9 for H(2), H(5), and H(6) with Pr. These metals are inefficient at relaxation due to an extremely short electron-spin relaxation time.[3,53] $T_{1(B)}$ values are certainly long compared to 10^{-4} sec (an upper bound for τ_M), so distance ratios can be estimated from the Pr data: $r_2/r_5 \sim 1.19$ and $r_6/r_5 \sim 1.24$. These can be compared with ratios calculated from molecular coordinates of 1.22 and 1.38, respectively. All the relaxation values are uncorrected for the effects of dissolved oxygen and, hence, represent lower bounds to the actual distance ratios. They are consistent with the Gd T_1 results.

Chemical shifts are found in Table I. These were also measured using a constant total lanthanide titration, essentially a variation of the fraction of total metal which is paramagnetic. Figure 2 shows three representative spectra The data were analyzed using a four-site fast-exchange shift

$$\omega_{obsd} = \omega_L P_L + \omega_{HL} P_{HL} + \omega_{LaL} P_{LaL} + \omega_{ML} P_{ML} \tag{18}$$

using the ω_L and ω_{HL} values obtained from the pH titration of ANTE alone, and P values calculated from the known stability constants. A simpler linear function of fraction bound

$$\omega_{obsd} = \omega_0 + P_B \omega_B \tag{19}$$

was also used. Under our conditions of constant total lanthanide, the fitted value of ω_B should be very close to $\omega_{ML} - \omega_{LaL}$; it represents the increase in shift obtained by replacing La(III) by a paramagnetic metal. This expected equality was found to be true, and all values in Table I are those obtained using eq 19.

The data are linear with fraction bound except for an initial nonlinearity at $P_B < 0.02$. This behavior was not seen in the Gd T_1 data, which was at much lower fraction bound, and could be accounted for by the four-site function (eq 18). The problem arises both from the inequivalence of M and La (stability constants for Eu and Pr are twice that of La) and mostly due to the difference in shift between LaL and HL. The net effect is an additional downfield shift at low fractional bound, which adds to the paramagnetic shift. It causes a lag in the Eu shifts and an increase in the Pr shifts. The simple linear function (eq 19) obviously cannot reproduce this behavior, and hence points at $P_B < 0.02$ were not used with this function. This nonlinearity does not appear in the T_1 experiments since LaL, HL, and L have the same T_1 values, and hence the observed $1/T_1$ increase is due strictly to the fraction bound to the paramagnetic metal.

The α and β,β' regions were examined using WEFT.[27] Simulations of the ABX multiplet structure showed that the relative bound shifts of β and β' induced by Pr or Eu are the same to within 0.02 ppm. The spectra have $J_{AB} \sim \delta_{AB}$ and, hence, the multiplet structure is very sensitive to small changes in δ_{AB}. To within experimental error the β and β' protons have the same induced shift. The simulated values of the coupling constants ($J_{\beta,\beta'} = -13.9$ Hz, $J_{\alpha,\beta} = 6.6$ Hz, $J_{\alpha,\beta'} = 7.9$ Hz) are in reasonable agreement with the values reported for the α,β,β' region of phenylalanine and tyrosine at high pH.[37,38] The interpretation of the lanthanide-induced shifts will be discussed at length below.

Experiments were attempted with Yb(III), but the line broadening was so severe that the lines were effectively obliterated after a very small amount of Yb(III) was added.

Line widths were estimated for the samples used to measure the chemical shifts by treating each of the ring proton multiplets as a sum of two [H(2) and H(5)] or four [H(6)] Lorentzian lines all of the same width. The observed line width data was fit to a quadratic function[39] of P_B

$$\pi\Delta\nu = \frac{1}{T_2} = \frac{P_A}{T_{2A}} + \frac{P_B}{T_{2B}} + \tau P_A P_B \Delta^2 \tag{20}$$

where A refers to the free species (i.e., not bound to paramagnetic) and B refers to bound, and τ is the geometric

Table III. Line Width Data

	$1/T_{2A}$[a]	$\tau\Delta^2$[b]	$\left[\frac{\tau\Delta^2_i}{\tau\Delta^2_5}\right]^{1/2}$	ω_i/ω_5[c]	$(r_i/r_5)^6$[d]
H(2)[e]	5.8	16.3	0.121	0.235	0.297
H(5)[e]	4.3	1115	(1)	(1)	(1)
H(6)[e]	4.4	188	0.411	0.404	0.161
H(2)[f]	5.3	6.8	0.197	0.177	0.297
H(5)[f]	2.2	175	(1)	(1)	(1)
H(6)[f]	2.4	64	0.605	0.535	0.161

[a] Line width in absence of broadening due to paramagnetic effects of the metal, in sec^{-1}. These vary with pH. See text. [b] In sec^{-1}. [c] From Table I. [d] Assuming oxygen–metal bond lengths of 2.4 Å. [e] Pr. [f] Eu.

mean exchange lifetime defined by

$$\tau = \frac{\tau_A\tau_B}{(\tau_A + \tau_B)} = \tau_B(1 - P_B) \qquad (21)$$

where τ_A is the first-order lifetime before exchange out of site A. One could expand τ as above and use a cubic fit to P_B, but due to scatter in the data, a four-parameter fit is not justified, and in any case the cubic term will be negligible; the line width data cover roughly P_B = 0–0.1, so the cubic term is $O(10^{-3})$. For a $\tau\Delta^2$ term of 1000, this only amounts to 1 sec^{-1} compared to the linear term which would be 100 sec^{-1}. Rearranging eq 20

$$\frac{1}{T_2} = \frac{1}{T_{2A}} + P_B\left(\frac{1}{T_{2B}} - \frac{1}{T_{2A}} + \tau\Delta^2\right) - P_B{}^2\tau\Delta^2 \qquad (22)$$

At the correlation times involved here (the electron-spin relaxation times of Pr and Eu) $1/T_{2B} = 1/T_{1B}$, and the $1/T_{1B}$ value from Pr and Eu relaxation data was used to specify $1/T_{2B}$. The data were analyzed by a two-parameter fit to $1/T_{2A}$ and $\tau\Delta^2$. The results are in Table III.

Comparison of Tables I and II will show $(1/T_2)_{\text{free}} > (1/T_1)_{\text{free}}$. This is due to exchange broadening from the equilibrium between protonated and deprotonated nitrotyrosine molecules. Analysis of this exchange shows that a forward rate of protonation of 2 × 10^9 M^{-1} sec^{-1} can account for the observed broadening, and this is quite reasonable for a diffusion-limited rate.

For a given metal, the ratio of the exchange-broadening terms should vary as the square of the bound shift. Hence, one can estimate shift ratios from line width data. Columns three and four compare the shift ratios calculated from line width and shift data. For comparison, the ratio of sixth power distance is also given. If the broadening was due to the bound relaxation time, then it would vary as column five. It clearly does not, and the broadening is therefore due to chemical exchange, as has been observed for organic shift reagents.[40] Columns three and four agree well except for Pr H(2), which is the most unreliable estimate due to a small change in line width.

Finally, as a further consistency check, the bound lifetime $\tau_B(\equiv\tau_M)$ can be estimated. Using the data for H(5), where the line broadening is the largest, and hence easiest to measure with reasonable accuracy, $\tau_M \simeq 1.1 \times 10^{-5}$ for Pr and 9×10^{-6} for Eu using $P_A = 0.9$. From the pressure jump rate constants and the stability constants of Table I, τ_M is calculated to be 6×10^{-6} and 6.5×10^{-6}, respectively, in reasonable agreement with the line width data.

Discussion

The comparison of the experimental lanthanide-induced shifts with theory requires the calculation of a function of the form

$$\left(\frac{\Delta\omega}{\omega_0}\right) = r^{-3}[K_1(3\cos^2\theta - 1) + K_2(\sin^2\theta\cos 2\phi)] \qquad (23)$$

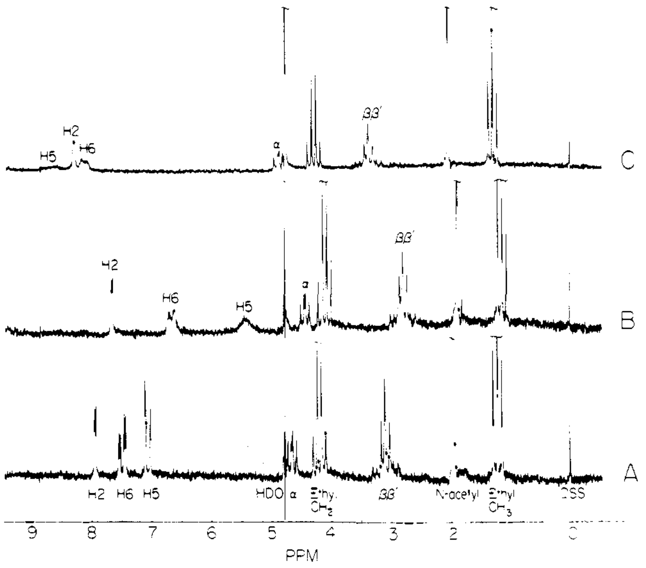

Figure 2. 100-MHz Fourier transform spectra of metal–ANTE solutions using a WEFT sequence to reduce the HDO line. The horizontal scale is in parts per million downfield from internal DSS. All solutions contained 9.59 mM ANTE, 0.1 M KCl in D_2O at pH 6.28, with variable lanthanide metal. 128 sequences of form PD-π-τ-π/2-AT were recorded for each spectrum. With times in seconds and concentrations in millimolar, the conditions for each spectrum are A, La(III) 14.24, PD 9.0, τ 5.5, AT 2; B, Eu(III) 8.14, La(III) 6.10, PD 6.5, τ 4.5, AT 2; C, Pr(III) 3.0, La(III) 11.3, PD 9.7, τ 5.0, AT 2. The vertical scale of C is half that of A and B.

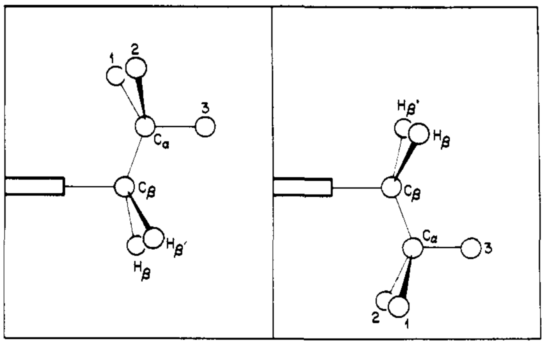

Figure 3. Internal conformers used in conformational averaging. The rectangle at the left of each figure is the ANTE ring oriented with the nitro group coming out of the page. The right and left figures differ by a 180° rotation about the ring C_1–C_β bond, holding the C_α–C_β conformation constant. Atoms 1, 2, and 3 are the cyclic permutations of (α-carboxyl carbon, α-amino nitrogen, α-hydrogen).

where the coordinate system and the coefficients K_1 and K_2 must be determined. In addition, the interaction must be averaged over any stereochemical conformers.[41] Thus, analysis of the α and β,β' shifts requires specification of the side-chain conformers. A space-filling model was constructed to aid in deciding which forms would most likely contribute. These are summarized in Figure 3. There are two configurations about the C_1–C_β bond, related by a 180° rotation about the bond axis. Further, there are three orientations about the C_α–C_β bond in which the atoms labeled 1, 2, and 3 are the three cyclic permutations of α-carboxyl carbon, α-amino nitrogen, and α-hydrogen, giving a total of six conformers. These choices are supported both by conformational calculations[42] and NMR coupling constant data[37,38] for the side chains of aromatic amino acids and are also consistent with the Gd T_1 data placing the ethyl and N-acetyl groups 8–10 Å from the metal. This implies there is no interaction of metal with the carbonyl oxygens of the blocking groups.

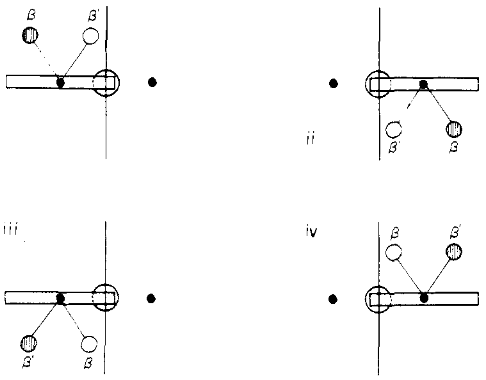

Figure 4. Schematic drawing of a "reflection set". Each figure is viewed down the C_β-C_1 bond. The large circle represents the metal, flanked by the coordination oxygens, which are symbolized by the black dots. The vertical line represents the pseudo-mirror plane. Pairs (i, ii) and (iii, iv) are "stereomers", differing by the sense of the coordination of the ANTE molecule to the metal. Pairs (i, iii) and (ii, iv) are reflections through the ANTE ring plane. As can be seen pictorially the conformers (i, iv) and (ii, iii) are equivalent. Labeling the reflection set to represent the conformers of Figure 3, the β and β' protons are therefore equivalent with respect to magnetic interactions with the metal.

The known X-ray structures of several lanthanide chelates show them to be nine-coordinate face-centered trigonal prisms with small distortions. The oxygens on the corners of the prism are at about 2.4 Å and those at the faces are 2.5 Å from the metal. Hence, for a bidentate ligand there are three unique ways to coordinate to the metal: (a) both oxygens at corners, (b) one at corner, the other at a face, and (c) both at faces, with oxygen-oxygen separations of 2.67, 2.86, and 4.33 Å, respectively. The nitrophenol O-O separation is 2.64 Å from the X-ray structure[34] and an increase would cause large strain. Hence, the corner-corner coordination is assumed. It is possible that other structures exist in solution, particularly eight coordinate square prisms and square antiprisms. The requirement for the analysis presented below to apply is that the plane bisecting the nitrophenol O-M-O triangle is a mirror plane with respect to the remainder of the coordination sphere.

An INDO-AFAOS molecular orbital calculation[43] on the nitrophenol anion was kindly performed by Dr. R. R. Birge, and the results showed the negative charge was delocalized around the ring in alternating fashion, with the bulk of the charge residing on the phenol and nitro oxygens: −0.53 and −0.46, respectively. Using similar methods, the H_2O oxygen is found to have a charge of −0.288 by comparison.[44] This indicates that the symmetry is destroyed by the nitrophenol anion, even if only the oxygens of the primary coordination sphere are considered. In the strictest sense, there is a complete loss of symmetry, but if the difference between the nitro and phenol oxygens is considered small, the XZ plane of Figure 1 is an approximate mirror plane.

Consideration of the $Ln(H_2O)_n(ANTE)^{2+}$ complex will show that there are two distinct orientations of the nitrophenol in the coordination sphere, not interconvertible by rotation of the molecule as a whole. They may be generated by rotating the ANTE molecule by 180° about the X axis of Figure 1 while maintaining the internal configuration of the ANTE rigid. Due to the fact that the metal coordination sphere is not symmetric with respect to the plane of the nitrotyrosine ring, these two configurations are not equivalent. Thus each internal conformer generates a pair of "stereomers", whose coordination spheres differ by reflection through the XZ pseudo-mirror plane. These stereomers are shown schematically in Figure 4 (i and ii) as viewed down the $C_\beta \rightarrow C_1$ bond.

Choosing the initial coordinate system with the Z axis along the X axis of Figure 1, Y axis as in the figure, and the X axis pointing into the plane of the paper, shifts are calculated using eq 10. For a given configuration, its stereo pair is generated by a rotation of the molecule by π about the Z axis, i.e., $C_{2m}(\theta,\phi) \rightarrow C_{2m}(\theta,\phi + \pi)$. In addition, the principal axes and hence the transform are reflected through the XZ plane. Reflection of the principal axis is equivalent to reflection of the *point* in the original axes, an operation achieved by replacing $C_{2m}(\theta,\phi)$ by $C_{2m}(\theta, -\phi)$. Hence the net effect is that the $C_{2m}(\theta,\phi)$ are transformed to $C_{2m}[\theta,-(\phi + \pi)] = C_{2m}(\theta,-\phi - \pi) = C_{2m}(\theta,\pi - \phi)$ since the functions are periodic in ϕ with period 2π.

The second operation is that of reflection through the YZ plane which simply replaces ϕ by $\pi - \phi$. So, to calculate the shift of a point which is the reflection of a point (θ,ϕ) through the ring plane, repeat the summation of eq 10 using $C_{2m}(\theta,\pi - \phi)$. Comparison with the result above shows that they are identical. Since the ring is in the YZ plane, the shift of a stereo pair is equivalent to the shift of a point reflected through the ring plane. Hence conformers ii and iii, i and iv form equivalent pairs.

A corollary of the above result is that the ring protons are unaffected by the stereoisomerism. This is seen easily by noting that the ring plane is defined by setting $\phi = \pm\pi/2$. The spherical functions are $C_{2m}(\theta,\pm\pi/2)$ and those of the stereo pair are $C_{2m}[\theta,\pi - (\pm\pi/2)] = C_{2m}(\theta,\pm\pi/2)$ and hence stereoisomerism does not alter the calculated shift for ring protons. It is important to note that these results are independent of the axis transform $\boldsymbol{D}^2(\alpha,\beta,\gamma)$ and hence are independent of the principal axis directions of the complex.

A prediction of the above considerations is that the β and β' protons will have the same induced shift. This is shown schematically in Figure 4. Conformers (i, ii) and (iii, iv) are stereo pairs as defined above, whereas (i, iii) and (ii, iv) are pairs related by an *internal* rotation of 180° about the C_1-C_β bond axis. The position of β' in iii is the *reflection* of β in i and vice versa. Similar relations hold between β and β' in ii and iv. Assuming equal populations of the four species, the net shift will be the average of the shifts of the individual conformers. For β

$$\omega(\beta) = [\beta(\mathrm{i}) + \beta(\mathrm{ii}) + \beta(\mathrm{iii}) + \beta(\mathrm{iv})]/4 \qquad (24)$$

where $\beta(x)$ represents the shift of β in conformer x. By the symmetry discussed above $\beta'(\mathrm{i}) = \beta(\mathrm{iv})$, $\beta'(\mathrm{ii}) = \beta(\mathrm{iii})$, $\beta'(\mathrm{iii}) = \beta(\mathrm{ii})$, and $\beta'(\mathrm{iv}) = \beta(\mathrm{i})$. So $\omega(\beta') = [\beta(\mathrm{iv}) + \beta(\mathrm{iii}) + \beta(\mathrm{ii}) + \beta(\mathrm{i})]/4 = \omega(\beta)$. This result is also independent of the actual axis system and only depends upon equality of conformer populations. Note that simple rotation about the C_1-C_β bond will not make β and β' equivalent, as is seen clearly in the NMR spectra of Figure 2. The observed induced shifts are the same to within 1%, and since the calculated difference between β and β' in absence of conformational averaging is more than 10%, the assumptions outlined above are experimentally verified.

The actual analysis of the data was performed by starting in a coordinate system with the Z axis along the C_3 axis of the 9 coordinate aquocation, obtained from that of Figure 1 by a rotation $R(0, \pi - 1.1735, \pi)$. A program COORD,[45] recoded in BASIC, was used to calculate the Cartesian coordinates of all the atoms. A rotation matrix was calculated using Euler angles α, β, γ to rotate these coordinates. From the rotated coordinates, the geometrical functions of eq 23 were calculated including conformational averaging and trial shifts calculated. These were compared to the observed shifts for H(2), H(5), H(6), and β,β'. From the theory sec-

Table IV. Best Fit Dipolar Parameters and Calculated Shifts[a]

	Pr(III)				Eu(III)			
	I	II	III	IV	I	II	III	IV
K_1	−641	997	−2152	−634	−275	−401	1191	83.1
K_2	−1511	−3460	(0)	−1519	1466	−2838	(0)	924
α	(0)	(0)	0.832	(0)	(0)	(0)	1.274	(0)
β	(−1.17)	(0)	−0.936	(−1.17)	(−1.17)	(0)	−1.065	(−1.17)
γ	−1.05	−0.659	(0)	(−1.05)	−1.18	0.623	(0)	(−1.05)
s				−0.097				0.995
	A	B	C	D	A	B	C	D
H(2)	4.00	3.98	3.98	0.034	−1.31	−1.25	−1.32	−0.34
H(5)	17.05	17.06	17.03	−0.04	−7.42	−7.45	−7.43	0.4
H(6)	6.88	6.80	6.88	0.08	−3.97	−3.73	−3.97	−0.8
α	2.56	2.50	2.50	(0)	−1.25	−1.51	−1.09	(0)
β,β'	2.98	3.12	3.11	(0)	−1.38	−1.76	−1.32	(0)
R		0.0029	0.0025			0.1250	0.0183	

[a] Parameters defined in text. Shifts are in parts per million from LaANTE. Angles are in radians, with positive rotations defined by the direction of advance of a right-hand screw along the particular axis. R is from ref 48. I, fixing principal Z axis normal to ring plane. II, fixing principal Z axis along C_3 axis of aquocation. III, pseudo-axial. IV, estimated contact term included. A, observed shifts from Table I. B, calculated using parameters of column I, II, or III. C, calculated using parameters of column IV. D, contact contribution to calculated shifts of column C.

tion, specification of two parameters will generate a unique solution. For the nonaxial fits, the polar angles α and β were specified, fixing the Z axis. The third angle γ and the constants of eq 23 were varied to fit the data. For the pseudo-axial fits, γ and K_2 were set to zero, and α, β, and K_1 were fit. The results of the fits are in Table IV. In all cases variation of parameters was done by a nonlinear least-squares program. Three cases for the nonaxial fits were considered: (a) the Z axis normal to the plane of the ring ($\alpha = 0$, $\beta = -1.1735$); (b) the Z axis along the C_3 of the aquocation ($\alpha = 0$, $\beta = 0$); (c) the Z axis is in the ring plane ($\alpha = 0$, $\beta = 0.3973$). Case a gives very close agreement for the coordinate systems of the Eu and Pr complexes (γ within 0.15 radian), and the X and Y axes are approximately bisected by the pseudo-mirror plane. Case b gives widely differing values for the coordinate systems for Pr and Eu, and case c gives physically unreasonable solutions ($A_2 \gg A_1$). The pseudo-axial Z axis is not at an apparent unique molecular axis, and further it is quite different for Pr and Eu. In light of the above, case a is the most reasonable solution since it gives the closest agreement between the Pr and Eu coordinate systems and bests reflects local symmetry. Note that in all cases the calculated shifts are *identical*, as expected, so they cannot be distinguished on the basis of the precision of the fit. It must be emphasized that the idea here was not to determine the susceptibility anisotropies but rather to arrive at operational parameters for use when nitrotyrosine is incorporated into a protein.

Examination of the results shows that the Pr data are fit very well by the dipolar model, but there are discrepancies with the Eu data. The most questionable assumption is the neglect of a contact interaction for the aromatic protons. The contact shift is given by[46]

$$\frac{\Delta H}{H} = \left(\frac{\Delta\omega}{\omega_0}\right) = \frac{A}{h}\frac{\langle S_z \rangle}{\gamma_N} \tag{25}$$

Using the upper bound of the Gd shift, and published data on the expectation value of $\langle S_z \rangle$ for the $4f^n$ ions at 300°K,[47] one finds that if the shift of Gd is $<\pm 3.4$ ppm then Eu $< \pm 1.1$ ppm and Pr $< \mp 0.4$ ppm assuming A, the hyperfine coupling constant, does not change. With no direct way of measuring the hyperfine coupling constant, the contact shift cannot be calculated. To estimate it, a crude correction was attempted by assuming that the magnitude of the contact shift was proportional to the charge density on the aromatic hydrogens obtained from the INDO-AFAOS calculation, taking into account the expected alternation in the sign of the spin density. The coordinate system was fixed to that of Pr with Z normal to the ring ($\alpha = 0$, $\beta = -1.1735$, $\gamma = -1.05$), and the data were fit to K_1, K_2, and s, the scaling factor of the contact shift. As column C of Table IV indicates, this procedure results in a great improvement in the fit to the europium data, while introducing almost negligible changes in the Pr fit. For reference, the R factor of Davis and Willcott[48] is tabulated below each column of calculated shifts. Note that the approximate contact corrections have opposite signs as expected,[47] but relative to the Eu values, the Pr corrections are low. This is probably due both to the errors in the above approximation as well as the insensitivity of the Pr fits to such a small correction. We emphasize that these corrections are only meant to be qualitative, i.e., to demonstrate that a reasonable contact model could explain the discrepancies between the dipolar and observed shifts for Eu.

Table V. Observed Shift Ratios[a]

	H(2)	H(5)	H(6)	α
Eu	0.949	5.38	2.88	0.91
Pr	1.34	5.72	2.31	0.84

[a] Ratios to the shift of the β protons.

Table V lists the observed shift ratios. These are referenced to the β protons due to their expected independence of a contact interaction. If the experimental error were large, it would be possible to call these ratios "constant" and erroneously conclude that the axial term was dominant. The results of the theory indicate, however, that a very strict definition of constant (probably less than a few percent variation) must be adhered to. The observed differences *are* significant compared to experimental error, indicating that axial symmetry does not exist. This is supported by the fact that the pseudo-axial transforms are not identical and that the physically reasonable axis system gives large nonaxial terms.

Conclusion

Our conclusions are as follows.

(1) It is not possible by solution NMR to unambiguously determine the principal axis of the lanthanide complex. Recently workers[49] have proposed variation of the orientation of the "principal axis" from the lanthanide donor bond. If the resulting axis lies close to the lanthanide donor bond,

and the data are fit well with the $C_{20}(\theta,\phi)$ factor alone, then axial symmetry is probably present, arising out of internal rotation[21] or stereochemical rearrangement.[50] If, however, the resulting axis bears no relation to the unique axes of the coordination sphere, then one is probably doing nothing more than locating the pseudo-axial system. Axial symmetry most likely does not exist despite the apparent fit of the data. This problem is expected to be all the more serious in the case of bidentate ligands like ANTE or possible coordination sites in proteins where the lanthanide donor bond is no longer unique and where rotational averaging is not possible. As we have seen for ANTE, the physically reasonable axes lead to axially asymmetric X tensors.

(2) Observation of constant internal shift ratios does not guarantee axial symmetry unless the deviations are very small. It may serve as an indicator of the presence of contact shifts, since when these are present, there may be no choice of axes which can give constant ratios. It would also indicate that the coordination sphere has not altered drastically from metal to metal.

(3) Using line width data to get r^6 information will be incorrect except for Gd(III). For the other metals, the line width is most likely dominated by chemical-exchange broadening and therefore reflects the square of the bound shift.

(4) Contact interactions, while small for protons, in general should not be dismissed a priori. We and others[3,51,52] have used the shifts induced by Gd(III) (which cannot induce dipolar shifts in solution) to estimate or at least bound the contact term. With an estimation from Gd(III), the contact terms for the other metals can be estimated from the known $\langle S_z \rangle$ values, assuming the hyperfine coupling constant is constant across the series. This effect is obviously more dependent on the degree of covalency present, as well as the nature of the ligands. Ligands with π systems accessible to the metal are expected to have more contact interaction possible.

(5) One point tacitly assumed in all of the above work (and by other workers as well) is that the replacement of the metals in the complexes is isomorphous; the coordination sphere and placement of all atoms relative to one another remain constant. Only the number of 4f electrons changes. The pressure jump data cited above, however, noted that the coordination number of the aquocations appeared to change in solution as one proceeds from La(III) to Lu(III). This was evidenced by the change in the forward ligation rate constant and indicates a constant coordination sphere from La to Gd, altering thereafter. Presumably the coordination drops from 9 in the light lanthanides to 8 in the heavier ones due to the smaller ionic radius and, hence, smaller available space in the primary coordination sphere. For those reasons we have chosen to work with the f^0–f^7 metals. Work in aqueous systems using the heavier metals should be approached with the thought of a changing coordination sphere in mind.

In spite of the difficulties cited above, we feel the lanthanide probe technique can provide valuable information about molecular structure in solution and can be useful in biological systems as well. Experiments on selectively nitrated derivatives of the basic pancreatic trypsin inhibitor have shown dramatic effects which can be ascribed to lanthanide binding at nitrotyrosine..

Acknowledgments. The authors wish to thank Dr. R. J. M. Anderson and Dr. R. R. Birge for very helpful discussions and the latter for performing the INDO-AFAOS calculation. We are also indebted to W. E. Hull for the use of his nonlinear least-squares program. The authors also wish to acknowledge the financial support of the National Institutes of Health (Grant GM-17190) and the National Science Foundation (Fellowship to G.S.).

References and Notes

(1) A. F. Cockerill, G. L. O. Davies, R. C. Harden, and D. M. Rackham, *Chem. Rev.*, **73**, 553 (1973).
(2) R. E. Sievers, Ed., "Nuclear Magnetic Resonance Shift Reagents", Academic Press, New York, N.Y., 1973.
(3) C. D. Barry, J. A. Glasel, R. J. P. Williams, and A. V. Xavier, *J. Mol. Biol.*, **84**, 471 (1974), and references cited therein.
(4) C. M. Dobson, R. J. P. Williams, and A. V. Xavier, *J. Chem. Soc., Dalton Trans.*, 1762 (1974) and references cited therein.
(5) C. D. Barry, J. A. Glasel, A. C. T. North, R. J. P. Williams, and A. V. Xavier, *Biochim. Biophys. Acta*, **262**, 101 (1972).
(6) V. F. Bystrov, N. I. Dubrovina, L. I. Barsulkov, and L. D. Bergelson, *Chem. Phys. Lipids*, **6**, 343 (1971).
(7) R. J. Kostelnik and S. M. Castellano, *J. Magn. Reson.*, **7**, 219 (1972).
(8) R. Jones, R. Dwek, and S. Forsen, *Eur. J. Biochem.*, **47**, 271 (1974).
(9) I. D. Campbell, C. M. Dobson, R. J. P. Williams, and A. V. Xavier, *Ann. N.Y. Acad. Sci.*, **222**, 163 (1973).
(10) P. M. Colman, L. H. Weaver, and B. W. Matthews, *Biochem. Biophys. Res. Commun.*, **46**, 1999 (1972).
(11) A. Levitzki and J. Reuben, *Biochemistry*, **12**, 41 (1973).
(12) D. W. Darnall and E. R. Birnbaum, *J. Biol. Chem.*, **245**, 6484 (1970).
(13) L. C. Sieker, E. Adman, and L. H. Jensen, *Nature (London)*, **235**, 40 (1972).
(14) J. F. Riordan and M. Sokolovsky, *Acc. Chem. Res.*, **4**, 353 (1971).
(15) B. Bleaney, *J. Magn. Reson.*, **8**, 91 (1972).
(16) W. D. Horrocks, *Inorg. Chem.*, **9**, 690 (1970).
(17) A. Carrington and A. D. McLachlan, "Introduction to Magnetic Resonance", Harper and Row, New York, N.Y., 1967.
(18) D. M. Brink and G. R. Satchler, "Angular Momentum", 2nd ed, Oxford Press, Oxford, 1968.
(19) W. D. Horrocks, J. P. Sipe III, and D. Sudnick in "Nuclear Magnetic Resonance Shift Reagents", R. E. Sievers, Ed., Academic Press, New York, N.Y., 1973.
(20) A. J. Graffeo and J. L. Bear, *J. Inorg. Nucl. Chem.*, **30**, 1577 (1968), and references cited therein.
(21) J. M. Briggs, G. P. Moss, E. W. Randall, and K. D. Sales, *Chem. Commun.*, 1180 (1972).
(22) R. H. Newman, *Tetrahedron*, **30**, 969 (1974).
(23) H. M. Irving and H. S. Rossotti, *J. Chem. Soc.*, 2904 (1954).
(24) For a discussion of metal-ligand equilibria, see S. Chabarek and A. E. Martell, "Organic Sequestering Agents", Wiley, New York, N.Y., 1959.
(25) L. G. Sillen and A. E. Martell, "Stability Constants of Metal Ion Complexes", The Chemical Society, London, 1971.
(26) C. Postmus, Jr., L. B. Magnusson, and C. A. Craig, *Inorg. Chem.*, **5**, 1154 (1966).
(27) S. L. Patt and B. D. Sykes, *J. Chem. Phys.*, **56**, 3182 (1972).
(28) Z. Luz and S. Meiboom, *J. Chem. Phys.*, **40**, 2686 (1964).
(29) A. Abragam, "The Principles of Nuclear Magnetism", Oxford Press, Oxford, 1961, Chapter 8.
(30) D. R. Fitzwater and R. E. Rundle, *Z. Kristallogr.*, **112**, 362 (1959).
(31) I. Grenthe, *Acta Chem. Scand.*, **23**, 1253, 1752 (1969).
(32) I. Grenthe, *Acta Chem. Scand.*, **25**, 3347 (1971).
(33) H. Steinfink and G. D. Brunton, *Inorg. Chem.*, **9**, 2112 (1970).
(34) J. P. G. Richards, *Z. Kristallogr.*, **116**, 468 (1961).
(35) G. Geier, *Ber. Bunsenges. Phys. Chem.*, **69**, 617 (1965).
(36) R. A. Dwek, R. E. Richards, K. G. Morallee, E. Nieboer, R. J. P. Williams, and A. V. Xavier, *Eur. J. Biochem.*, **21**, 204 (1971).
(37) J. R. Cavanaugh, *J. Am. Chem. Soc.*, **90**, 4533 (1968).
(38) J. R. Cavanaugh, *J. Am. Chem. Soc.*, **92**, 1488 (1970).
(39) T. J. Swift and R. E. Connick, *J. Chem. Phys.*, **37**, 307 (1962).
(40) J. Reuben and J. S. Leigh, Jr., *J. Am. Chem. Soc.*, **94**, 2789 (1972).
(41) I. Armitage, L. D. Hall, A. G. Marshall, and L. G. Werbelow, *J. Am. Chem. Soc.*, **95**, 1437 (1973).
(42) P. K. Ponnuswamy and V. Sasisekharan, *Int. J. Protein Res.*, **3**, 9 (1971).
(43) R. R. Birge, *J. Am. Chem. Soc.*, **95**, 8241 (1973).
(44) J. A. Pople and D. L. Beveridge, "Approximate Molecular Orbital Theory", McGraw-Hill, New York, N.Y., 1970, p 91.
(45) R. Rowan, Thesis, Harvard University, 1974.
(46) N. Bloembergen, *J. Chem. Phys.*, **27**, 572, 595 (1957).
(47) R. M. Golding and M. P. Halton, *Aust. J. Chem.*, **25**, 2577 (1972).
(48) R. E. Davis and M. R. Willcott, *J. Am. Chem. Soc.*, **94**, 1744 (1972).
(49) G. E. Hawkes, D. Liebfritz, D. W. Roberts, and J. D. Roberts, J. *Am. Chem. Soc.*, **95**, 1659 (1973).
(50) W. D. Horrocks, *J. Am. Chem. Soc.*, **96**, 3022 (1974).
(51) G. LaMar and J. W. Faller, *J. Am. Chem. Soc.*, **95**, 3817 (1973).
(52) K. Ajisaka and M. Kainosho, *J. Am. Chem. Soc.*, **97**, 330 (1975).
(53) A. Abragam and B. Bleaney, "Electron Paramagnetic Resonance of Transition Ions", Oxford University Press, Oxford, 1970.