LANTHANIDE INDUCED NMR PERTURBATIONS OF HEW LYSOZYME: EVIDENCE FOR NONAXIAL SYMMETRY

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Summary. Attention has recently been focused on the use of the trivalent lanthanides ions as probes in NMR studies of macromolecules. Previous analyses of the shifts induced by these paramagnetic ions have assumed that the magnetic susceptibility tensor of the metals is axially symmetric. This study has tested the validity of this assumption by analyzing reported NMR shift and relaxation data for various lanthanide complexes with lysozyme. For the Gd^{3+}-lysozyme complex the broadening data, fit to a $(1/r^6)$ model, places the metal ion in close proximity to the reported x-ray position. Contrary to current assumptions, the shifts induced by Nd^{3+} and Ce^{3+} exhibit considerable non-axial character. On the basis of a statistical hypothesis test, the axially symmetric model was rejected with more than 97% confidence.

The controlled alteration of the NMR spectra of biomolecules by the addition of paramagnetic ions has continued to be a source of interest since the first report of Co^{2+} binding to lysozyme by McDonald and Phillips (1). Recently, lanthanide ions have served as probes in studies of biological molecules (2). Magnetic resonance studies and other spectroscopic investigations have demonstrated that these lanthanide ions can form strong and specific complexes with proteins and nucleic acids (3). In such paramagnetic macromolecular complexes, the electron-nuclear interactions produce two changes in the NMR spectra of the macromolecule: chemical shift perturbations and enhanced relaxation rates (2). Strategies for employing these changes in conjunction with structural information available from x-ray crystallographic techniques to determine solution conformations have appeared in the literature (4,5,6). These paramagnetic perturbations may also aid in the assignment

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of resonances in the NMR spectrum of the macromolecule provided that a theoretical understanding of the origin of these spectral changes can be reached.

Of the macromolecules studied to date, hen-egg-white lysozyme (EC 3.2.1.17) has been adopted as a model system for the development of the techniques necessary for the analysis of lanthanide-ion induced NMR spectral perturbations in terms of three-dimensional structure. This enzyme offers several clear advantages as a model system: it has been shown to bind polyvalent cations strongly (7,8); it is a low molecular weight enzyme with a known amino acid sequence (9,10); the mechanism of its enzymatic activity has been established (11,12); the x-ray crystallographically determined structure for native lysozyme (13-16) as well as Fourier difference maps for various metal-lysozyme complexes have been reported (8,16); and the lanthanide induced shifts for most of the lanthanides as well as relaxation data for gadolinium have been published (4-6).

The work presented here was undertaken as a first step in the development of a quantitative computer-based analysis of shift data for macromolecules which would be comparable to those methods which have been applied to smaller molecules in organic solution (17). As a starting point, we have analyzed the shift and relaxation data reported by R.J.P. Williams *et al.* (4,6) with a view to establishing the form of the dipolar shift equation (*vide infra*) that fits the data most precisely. In this treatment, we employed the x-ray structure reported by D.C. Phillips *et al.* (13,14,15), in conjunction with relaxation data from gadolinium (6) to establish the position of the Gd^{3+} ion in the metal-enzyme complex. This position was then used in analyzing the shift data reported for Nd^{3+} and Ce^{3+}. We present here the results of our analyses along with a method for testing the two forms of the dipolar shift equation (*vide infra*) based on a statistical hypothesis testing scheme.

Theory

The linewidth at half height ($1/T_{2M}$, in sec^{-1}) of a resonance in the gadolinium-lysozyme complex is given by (18)

$$1/T_{2M} = cf(\tau)/r^6 \qquad (1)$$

Where c is a constant which contains electronic terms of the gadolinium ion, $f(\tau)$ is a function of correlation times and r is the distance between the Gd^{3+} ion and the nucleus or nuclei being monitored.

The incremental shift observed in the presence of lanthanide ions can be expressed as (19)

$$\Delta\delta = \delta_C + \delta_D \qquad (2)$$

where δ_C is the shift arising from the Fermi contact interaction and δ_D is the shift arising from the dipolar (pseudocontact) interaction. Since δ_C arises from a through bond mechanism, and since, in macromolecules, the resonances being observed correspond to residues which are usually many bonds removed from the binding site, δ_D should be the dominant term in equation (2). General expressions for δ_D, have been derived and experimentally verified (20). The shift, δ_D, is given by

$$\delta_D = K_1[(3\cos^2\theta-1)/r^3] + K_2(\sin^2\theta\cos 2\phi/r^3) \qquad (3)$$

where K_1 and K_2 are constants which depend on the electronic quantum states of the individual lanthanide ion, and (r,θ,ϕ) are the spherical polar coordinates (referred to the principal magnetic axis system of the lanthanide) of the nucleus or nuclei associated with the shifted resonance. If $K_2 \neq 0$ equation (3) is referred to as the non-axial model; $K_2 = 0$ or $K_2 << K_1$ represents the axial model.

Analysis and Results

All computations were performed on an IBM 370 computer using a versatile least squares fitting algorithm written in Speakeasy (21). This algorithm employs the Marquardt search technique (22), which combines a steepest descent routine with a first-order Taylor's approximation. This method provides convergence even for arbitrary initial para-

TABLE 1

Data for Gd^{3+} and Nd^{3+} in HEW Lysozyme and Results of Best Fits of These Data

	Gd^{3+} in lysozyme				Nd^{3+} in lysozyme		
Resonance Observed	Reported Broadening[a]	Assumed σ	At minimum of fit (f-y)/σ[b]	r,A°	Reported Shift[2]	Assumed σ	At minimum (f-y)/σ
$Val^{109}C^{\gamma 1}H_3$	2300	Omitted from fit		8.81	-110	11	+0.72
$Val^{109}C^{\gamma 2}H_3$	2300	230	+0.46	6.02	-380	38	-0.29
$Ala^{110}CH_3$	1750	175	+0.88	6.26	64	6.41	+0.21
$Trp^{108}C^{\gamma}H$	1200	120	-0.95	6.87	-55	5.5	-0.09
$Ala^{31}CH_3$	163	16.3	-4.58	10.44	-69	omitted from fit	
$Thr^{51}CH_3$	140	14	+0.36	9.61	100	10	-0.10
$Tyr^{53}C^{\gamma 1}H$	100	14	-2.28	10.90	100	14	-2.63
$Tyr^{53}C^{\gamma 2}H$	100	14	+1.69	9.87		14	+1.40
$Leu^{56}C^{\gamma 1}H_3$	95	14	-3.83	11.85	12	7.7	+0.84
$Leu^{56}C^{\gamma 2}H_3$	95	14	+3.66	9.60		7.7	+0.79
$Ile^{98}C^{\gamma 2}H_3$	75	10	-5.45	13.3	-11	5.5	+0.74
$Met^{105}CH_3$	35	10	-0.58	12.6	5	5.5	-1.01
$Met^{12}CH_3$	25	10	-1.35	14.7	7	5.5	-0.49
$Leu^{17}C^{\gamma 1}H_3$	25	14	-1.47	17.2	12	7.7	-0.97
$Leu^{17}C^{\gamma 2}H_3$	25	14	1.26	15.8		7.7	-0.93

[a]Relative to $Tyr^{53}C^{\gamma}H$ as 100.
[b]For Gd^{3+}, f is given by equation (1) and y is the reported broadening.
[c]For Nd^{3+}, f is given by equation (3) and y is the reported shift.

meter estimates (steepest descent feature) as well as error estimates for the parameter values obtained at the best-fit convergence (Taylor's approximation). Convergence is considered to be complete when parameter values are stable to 1 part in 10^5.

The correspondence between observed and calculated values of either shift or line-broadening data was assessed using two statistical quantities. The first, called the "goodness of fit" parameter, χ^2, is defined by

$$\chi^2 = [1/(N_d - N_p)] \sum_{i=1}^{N_d} [(f_i - y_i)/\sigma^i]^2 \qquad (4)$$

where N_d is the number of data points fitted, N_p is the number of variable parameters, f_i is the value calculated at each point, y_i is the observed shift or broadening at each point and σ_i is the assumed error. The

second parameter, called the "agreement factor", R, is given by (23,24)

$$R = \sum_i \frac{f_i - y_i}{\sigma_i}^2 \Big/ \sum_i \frac{y_i}{\sigma_i}^2 \Big]^{1/2}$$

The use of the agreement factor as the basis for statistical hypothesis testing has been described by Hamilton (23) and Willcott et al. (24).

The shift (Nd^{3+}) and broadening data (Gd^{3+}) used in these computations are compiled in Table 1. In order to perform the computation, error estimates had to be assumed. Two approaches were used: a uniform error estimate of ± 10% was assumed for each literature value, or larger relative errors were assigned to the smaller observed values, which are expected to be less precise. Assumed values for the errors are listed in columns 3 and 6 in Table 1. In addition for data corresponding to two related resonances exhibiting the same shift or broadening, the associated errors were multiplied by 1.4 in order to halve the weight of each member of the pair in the least-squares sum. The resonances for which this adjustment was performed are indicated by braces in Table 1.

X-ray coordinates for native lysozyme (13,14) were used to generate the cartesian coordinates for the various hydrogens in space. The use of atomic coordinates of the native lysozyme is justified by x-ray data (8) which indicate that the conformational perturbations associated with metal binding to this enzyme are confined to the immediate vicinity of the metal binding site. Residues in close proximity to the metal binding site were excluded from this analysis. Methine and methyl hydrogen coordinates were calculated assuming a C-H bond distance of 1.09 A^o with approximate sp^2 and tetrahedral geometries, respectively. The methyl group was represented by the average position of the 3 hydrogens.

Relaxation Data

The Gd^{3+} data in Table 1 was fit to equation (1). This fitting procedure varied four parameters: the three cartesian coordinates of the Gd^{3+} in the crystal axis system and the project cf(τ). The different

TABLE 2

Statistics[a] and Parameters[b] for Lysozyme Shift Data

Model	χ^2	R Factor R	Ratio	$K_1(10^4 A^{o3})$	K_2/K_1	$\phi e(^o)$	$\theta e(^o)$	$\psi e(^o)$
Nd^{3+} Non-Axial (all data)	10.1	0.375	2.45	7.7±0.5	+0.80±0.04	163+2	123±2	1±2
Nd^{3+} Non-Axial ($Ala^{31}CH_3$ omitted)	1.6	0.153	2.63	6.0±0.4	+0.84±0.09	0+3	142±2	10±3
Nd^{3+} Axial[b] ($Ala^{31}CH_3$ omitted)	9.1	0.402		-5.3±0.3	-	19±1	144±1	-
Ce^{3+} Non-Axial ($Ala^{31}CH_3$ omitted)	2.2	0.188	2.42	5.2±0.5	+0.80±0.06	169±3	123±2	1±4
Ce^{3+} Axial[b] ($Ala^{31}CH_3$ omitted)	10.5	0.455		3.9±0.2	-	42±4	42±1	-

a) The error estimates are these generated by the Taylor's approximation in the least-squares algorithm.

b) Smaller of two observed minima.

fits were performed in order to test the sensitivity of the final parameter values to the assumed errors and to the omission of various residues from the fit (e.g. Val^{109} $C_\gamma{}^1H_3$). Inconsistences in parameter values were observed when the Val^{109} $C_\gamma{}^1H_3$ was included. On this basis, it was concluded that there was an inconsistency in the reported Val^{109} $C_\gamma{}^1H_3$ broadening. Results for the best fit of Gd^{3+} are given in Table 1. The final Gd^{3+} parameters:

(x = 8.4±0.2 A^o, y = 22.7±0.1 A^o, z = 16.9±0.4 A^o, $cf(\tau) = 1.14\pm0.22 \times 10^8$ A^{o6}) gave a χ^2 of 9.47. The Gd^{3+} position is in close proximity to the Gd^{3+} position (labeled (I) in reference (8)) reported from x-ray studies.

Shift Data

The shift data were fit to the two different forms of equation (3). For the axial model, 3 parameters were varied; K_1 and the two Euler

†These error limits reflect the variation in these parameters from fit to fit; they are not the errors generated in a single fit,

angles ϕ_e and θ_e (25), which specify the orientation of the magnetic axis relative to the crystal axis system. For the non-axial case, two additional parameters are required; K_2/K_1 and the third Euler angle, ψ_e. The results of these computations are summarized in Table 2.

For all of the non-axial fits, there are numerous sets of the five parameters which generate the same calculated values of the shifts. These include: 3 sets from an arbitrary labeling of the z-axis; for each of these, 2 sets from the direction of the z-axis; 4 sets arising from 90^o rotations of the x,y axes around the chosen z-axis; and finally two distinct sets of Euler angles which give identical rotation matrices. We have adopted the following convention in reporting the sets of parameter values summarized in Table 2: The z axis is chosen by restricting K_2/K_1 to satisfy $-1<K_2/K_1\leq 1$; the positive end of the z axis is directed in a particular orientation by requring $0\leq K_2/K_1\leq 1$. The two Euler angles were restricted in the following way: $0\leq\psi_e<90^o$; and $0\leq\theta_e<180^o$. Using these parameters, the dipolar shifts for the remaining residues of lysozyme can now be calculated. These predicted shifts may provide a method for the identification of nonassigned resonances in the lysozyme spectrum.

Two hypothesis tests were performed for Nd^{3+}. In all of the fits to the Nd^{3+} data, the $Ala^{31}CH_3$ shift showed the largest deviation from its calculated value. On the basis of the R-factor ratio obtained from the inclusion and omission of this resonance, we conclude with 99.5% confidence that the shift reported for the $Ala^{31}CH_3$ is inconsistent with the model. It was also clear that the axial model did not fit the data precisely. On the basis of the R-factor ratio obtained from the axial and non-axial fits, we conclude with 97.5% confidence that the assumption of axial symmetry for the magnetic susceptibility tensor of Nd^{3+} is untenable. This latter hypothesis test was also performed for Ce^{3+}. In this instance as well, the axial model can be rejected with 97.5% confidence.

These findings are in agreement with those reported by Reuben (26)

for the shifts induced by the neodymium-EDTA complex. Therefore, the analysis of the shifts induced by the trivalent lanthanides may require the non-axial model rather than the axial model. The establishment of the correct form of equation (1) is the first step in the analysis of the shifts induced by lanthanides in biomolecules in terms of structure, provided that the resonances of the biomolecule have been independently assigned. Application of this technique to various biomolecular problems is currently in progress in this laboratory.

REFERENCES

1. McDonald, C.C., and Phillips, D.C. (1969) Biochem. Biophys. Res. Commun. 35, 43-51.

2. For several reviews on this subject see:

   (a) Dwek, R.A. (1973) Nucl. Mag. Res. Biochem. (Oxford Press, London); (b) Nieboer, E. (1975) Structure and Bonding 22, Chapter 1.; (c) Levine, B.A., and Williams, R.J.P. (1975) Proc. R. Soc. Lond. A. 345, 5-20; (d) Reuben, J. (1975) Naturiviss 62, 172-178.

3. (a) Darnall, D.W., and Birnbaum, E.R. (1970) J. Biol. Chem. 245, 6404-6486; (b) Reuben, J. (1971) Biochemistry 19, 2834-2838; (c) Barry, C.D., North, A.C.T., Glasel, J.A., Williams, R.J.P., and Xavier, A.V. (1971) Nature Land 232 236-245; (d) Kayne, M.S., and Cohn, M. (1972) Biochem. Biophys. Res. Commun. 46, 199-2005; (e) Epstein, M., Levitzki, A., and Reuben, J. (1974) Biochemistry 13, 1777-1782.

4. Campbell, I.D., Dobson, C.M., Williams, R.J.P., and Xavier, A.V. (1973) Ann. N.Y. Acad. Sci. 222, 163-174.

5. Campbell, I.D., Dobson, C.M., and Williams, R.J.P. (1975) Proc. R. Soc. Lond. A. 345, 23-39.

6. Campbell, I.D., Dobson, C.M., and Williams, R.J.P. (1975) Proc. R. Soc. Lond. A. 345, 41-59.

7. Ikeda, K., and Hamaguchi (1973) J. Biochem. 73, 307-322.

8. Kurachi, K., Sieker, L.C., and Jensen, L.H. (1975) J. Biol. Chem. 250, 7663-7667.

9. Jolles, P., Juarigini-Adell, J., and Jolles, J. (1964) C.R. Acad. Sci. 258, 3926-3928.

10. Canfield, R.E., and Liu, A.K. (1965) J. Biol. Chem. 240, 1997-2002.

11. Rupley, J.A. (1967) Proc. Roy. Soc. Sci. B. 167, 416-428.

12. Smith, L., Mohr, L., and Raftery, M. (1973) J. Amer. Chem. Soc. 95, 7497-7500.

13. Blake, C.C.F., Koenig, D.F., Mair, G.A., North, A.C.T., Phillips, D.C., and Sarma, V.R. (1965) Nature 256, 757-761.

14. Phillips, D.C. (1967) Proc. Natl. Acad. Sci. USA 57, 484-495.

15. Imoto, I., Johnson, L.N., North, A.C.T., Phillips, D.C., and Rupley, J.A. (1972) in The Enzymes ed. Boyer, P.D. (Academic Press, New York) Vol. 7, 665-868.

16. Yonath, A., Smilansky, A., and Sharon, N. (1974) FEBS Letters 49, 178-180.

17. Willcott, M.R.W., III, and Davis, R.E. (1975) Science 190, 850-857, and references cited therein.

18. Reuben, J., and Leigh, J.S., Jr. (1972) J. Amer. Chem. Soc. 94, 2789-2793.

19. McConnell, H.M., and Robertson, R.E. (1958) J. Chem. Phys. 29, 1361-1365.

20. Derivations: (a) Bleaney, B. (1972) J. Magn. Res. 8, 91-100; (b) Golding, R.M., and Pyykko, P. (1973) Mol. Phys. 6, 1389-1396 (1973). Experimental Verification: (a) Bleaney, B., Dobson, C.M., Levine, B.A., Martin, R.B., Williams, R.J.P., and Xavier, A.V. (1972) J.C.S. Chem. Commun. 791-793; (b) Elgavish, G.A., and Reuben, J. (1974) J. Magn. Res. 16, 360-361; (c) Stont, E.W., Jr., and Gatowsky, H.S. (1976) J. Magn. Res. 24, 389-398.

21. Cohen, S. (1976) Amer. Fed. of Infor. Processing Societies Nat'l Computer Conf. Proc. (AFIPS press Montvale, N.J.) 45, 1039-1047.

22. Marquardt, D.W. (1963) J. Soc. Indust. Appl. Math. 11, 431-441.

23. Hamilton, W.C. (1965) Acta Cryst. 18, 502-510.

24. (a) Willcott, M.R.W., III, Lenkinski, R.E., and Davis, R.W. (1972) J. Amer. Chem. Soc. 94, 1742-1744; (b) Davis, R.E., and Willcott, M.R., III, (1972) J. Amer. Chem. Soc. 94, 1774-1775.

25. Goldstein, H. (1959) Classical Mechanics (Addison-Wesley, Reading Mass.) 107-109.

26. Reuben, J. (1977) J. Amer. Chem. Soc. 99. In Press.