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Nitrotyrosine Chelation of Nuclear Magnetic Resonance Shift Probes in Proteins: Application to Bovine Pancreatic Trypsin Inhibitor†

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ABSTRACT: The interactions of Pr(III) and Eu(III) with specifically nitrated derivatives of the basic bovine pancreatic trypsin inhibitor have been studied using optical spectroscopy and nuclear magnetic resonance (NMR) at 250 and 270 MHz. Stability constants for proton and metal binding to nitrotyrosines 10 and 21 determined optically are in good agreement with those from NMR. Observation of the Eu(III)-induced NMR shifts of the ring protons of nitrotyrosine 21 allowed calibration of the magnetic interactions for this binding site. The Pr(III)-induced shifts for several resolved nonexchangeable backbone proton resonances were compared with calculated shifts using the known x-ray structure. With several simplifying assumptions, the Pr(III)-induced shifts were used to assign one α-CH and five NH protons to compatible sets of backbone positions which are consistent with the known pH dependence and resistance to exchange with solvent D_2O. Some of the more general aspects of lanthanide-induced shifts are discussed with reference to their use in proteins. Due to the complexities of the analysis of the shift data, the most straightforward use of this technique is in conjunction with the relaxation probe Gd(III) for measurement of intramolecular distances.

Nitrotyrosine has been proposed as a binding site for lanthanide ions as nuclear magnetic resonance structural probes of proteins in solution (Marinetti et al., 1975). Specific tyrosine nitration can be used to introduce a binding site into a protein at a known locus, extending the use of the lanthanide NMR[1] probes to proteins which do not possess a naturally occurring binding site for the lanthanide ions. This approach has been successfully applied to a study of the basic pancreatic trypsin inhibitor using Gd(III)-induced NMR relaxation (Marinetti et al., 1976). The major interest in the lanthanide ions is that the through-space dipolar interaction seems to account for most of the observed shifts and relaxation. Because this has a known and reasonably simple dependence on the relative position of the nucleus and the metal (Horrocks et al., 1973; Bleaney, 1972), the lanthanide-induced effects contain information on the three-dimensional structure of the molecule in solution. Lanthanide studies with lysozyme have helped assign some protein resonances to specific side chains (Campbell et al., 1973, 1975). These and other biological applications of the lanthanides have been reviewed (Reuben, 1975).

This manuscript extends the previous studies of lanthanide interactions with nitrotyrosine 21 of dinitro-BPTI. Stability constants for metal binding have been determined optically as an independent check on the values derived from NMR. The shifts induced by Pr(III) and Eu(III), attributed to nitrotyrosine 21 bound metal, have been analyzed quantitatively using the nitrotyrosine 21 ring protons to calibrate the magnetic interactions. Although the induced shifts of the ring protons behaved qualitatively like the model system, *N*-acetyl-L-3-nitrotyrosine ethyl ester, there was a difference in magnitude in the induced shifts between the protein and the model system. The shift results for the nonexchangeable NH resonances of the protein can be used, after making several assumptions, to tentatively assign five of the NH resonances to compatible sets of backbone positions. The assignments are consistent with other data such as the known pH dependence and resistance to exchange with solvent D_2O of the NH protons. Finally, the requirements for and limitations on the application of the

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[1] Abbreviations used: NMR, nuclear magnetic resonance; UV, ultraviolet; ppm, parts per million; BPTI, bovine pancreatic trypsin inhibitor; ANTE, *N*-acetyl-L-3-nitrotyrosine ethyl ester; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-2-hydroxymethylpiperazine-*N*′-2-ethanesulfonic acid.

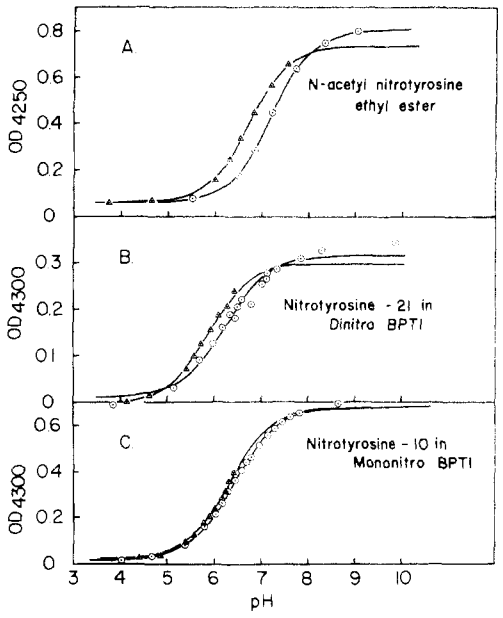

FIGURE 1: pH titrations of nitrotyrosines monitored by visible absorption. (A) ANTE (0.19 mM)–9.8 mM Hepes: (⊙) 0.12 M KCl; (▲) 0.1 M KCl, 4.8 mM $PrCl_3$. (B) Derived from observed data for 0.094 mM dinitro-BPTI in 15.6 mM Pipes, 0.1 M KCl, 0.25 mM DSS, and the solution of part C (see text): (⊙) no metal; (▲) 22 mM $GdCl_3$. (C) Mononitro-BPTI (0.188 mM) in the buffer of part B: (⊙) no metal; (▲) 22 mM $GdCl_3$. All solutions are in D_2O. The wavelengths employed correspond approximately to the absorption maximum of the anionic form. The solid lines are computer fits to a single titration curve.

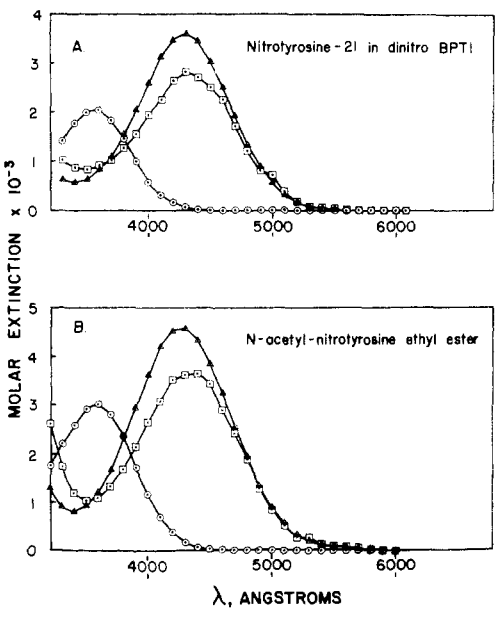

FIGURE 2: Visible spectra of nitrotyrosines. (A) Nitrotyrosine 21 derived from spectra of dinitro- and mononitro-BPTI. (B) ANTE. Solutions are as described in Figure 1. (⊙) Observed spectrum at low pH (A, pH 3.85; B, pH ~5); (▲) observed spectrum at high pH (A, pH 9.86; B, pH ~9); (⊡) calculated spectrum for the metal bound species from a spectrum with metal present at intermediate pH.

lanthanide probe technique to structural studies in proteins are discussed.

Materials and Methods

BPTI was obtained as a gift from Farbenfabriken Bayer AG (Elberfeld, Germany). Pipes and Hepes were from Calbiochem. D_2O was obtained from Bio-Rad; DCl and NaOD were from Stohler Isotope Chemicals. Lanthanide metals were purchased as the oxides from Johnson-Matthey and were Specpure grade. The preparation and handling of BPTI and its nitrated derivatives have been described previously (Snyder et al., 1975).

^1H NMR spectra were obtained at 250 MHz on the MPC-HF-250 superconducting spectrometer (Dadok et al., 1970) using correlation spectroscopy (Dadok and Sprecher, 1974). The 270-MHz ^1H NMR spectra were obtained on a Bruker HX-270. Acquisition conditions are as described previously (Marinetti et al., 1976).

Visible spectra of BPTI and derivatives were obtained on a Cary 14 spectrophotometer with matched quartz cells (Pyrocell Inc.), using a cell with buffer in the reference compartment. ANTE visible spectra were obtained similarly on a MacPherson-Heath spectrophotometer. pH was measured using an Ingold 14203 combination electrode using either a Beckman Expandomatic SS-2 or Corning Model 112 pH meter. All pH values are the uncorrected meter reading.

Optical Results

In the previous paper, estimates of the stability constants for lanthanide binding to the two nitrotyrosine side chains of a dinitrated derivative of BPTI were obtained from NMR measurements (Marinetti et al., 1976). To get an independent measurement of these stability constants, optical experiments were performed. They take advantage of the optical absorption of nitrotyrosines, which are well away from the UV absorptions of the rest of the protein, and hence give direct information about the local environment of these residues. The pH was varied in the presence and absence of a large excess of lanthanide metal, and the absorption was analyzed using eq A4 and A5 of Marinetti et al. (1976). These equations were derived for NMR chemical shifts, but the optical absorption data follow the same functional form, assuming Beer's law holds.

Figure 1 presents the titration data, taken at the absorption maximum of the anionic form. The nitrotyrosine 21 data were obtained by taking the difference between the observed spectra for dinitro-BPTI, and the calculated contribution of nitrotyrosine 10 obtained by analysis of the spectra for mononitro-BPTI. Protein concentrations calculated using the published extinction coefficients for nitrated BPTI derivatives (Meloun et al., 1968) were within experimental error of those obtained from the known weight of BPTI used to make the solutions.

The figure shows that, in the presence of lanthanide metals, the nitrotyrosine groups titrate at a lower pH than in the absence of metal. This difference in the titration behavior is related to the stability constant for metal binding. The data from this work and previous studies are summarized in Table I. Within experimental error, the paramagnetic lanthanides Eu(III), Pr(III), and Gd(III) behave similarly in binding strength with a given ligand. The weakened binding of both nitrotyrosines 10 and 21 compared with the model compound ANTE is seen in the optical and NMR data, with nitrotyrosine 21 binding tighter than 10.

Figure 2 presents observed optical spectra for the nitrophenol and nitrophenolate anion of nitrotyrosine 21 of dinitro-BPTI and ANTE. In addition, the spectrum of the lanthanide–nitrotyrosine complex was calculated from a spectrum with metal present at intermediate pH using the observed stability constants. Due to cumulative errors in the procedures used to correct for light scattering and more importantly in the stability constants for metal binding, the absolute magnitude of the absorption spectrum is subject to some uncertainty (±35%), but the general shape and position of the maximum

TABLE I: Summary of Binding Data.

Ring	Log K_a[a]	La(III) Log K_x[b]	La(III) Log K_1[c]	Pr(III) Log K_x[b]	Pr(III) Log K_1[c]	Eu(III) Log K_x[b]	Eu(III) Log K_1[c]	Gd(III) Log K_x[b]	Gd(III) Log K_1[c]	Av[k] Log K_1
N-Acetylnitrotyrosine ethyl ester	7.1[d]		2.3		2.6		2.6		2.5	2.6
	7.1[e]									
	7.2[f]	7.0	2.2	6.7	2.7					2.7
Nitrotyrosine 10	6.6[g]									
	6.6[h]					6.4	1.7			1.7
	6.4[j]							6.2	1.5	1.5
Nitrotyrosine 21	6.4[g]									
	6.5[h]			6.0[i]	2.2[i]					2.2
	6.3[j]							5.8	2.0	2.0

[a] Log proton stability constant with no metal present ± 0.1. [b] Log apparent proton stability constant in presence of metal ± 0.1. [c] Log metal-ligand stability constant ± 0.3 unless otherwise noted. [d] Data from potentiometric titration from Marinetti et al. (1975). Log K_1 are ±0.1. [e] NMR titration at 100 MHz from Marinetti et al. (1975). [f] Optical experiments, this paper. Average of data at 3575, 4250, and 4700 Å. [g] NMR titrations at 250 MHz from Snyder et al. (1975). [h] NMR titrations at 270 MHz from Marinetti et al. (1976). [i] Indirect measurements from effects on other resonances, 250 MHz from Marinetti et al. (1976). [j] Optical experiments, this paper. Average of data at 3500 and 4300 Å. [k] Average of data for Pr(III), Eu(III), and Gd(III).

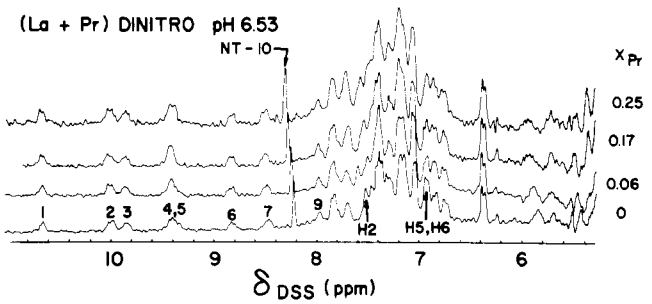

FIGURE 3: Pr(III) titration of dinitro-BPTI. The 250-MHz ^{1}H NMR spectra of the aromatic region. Resolved backbone NH's appear from 8 to 10.8 ppm, numbered sequentially. "NT-10" is the H2 resonance of nitrotyrosine 10. "H2, H5, H6" are the ring proton resonances of nitrotyrosine 21. Buffer is 15 mM Pipes, 0.5 mM DSS, 0.1 M KCl in D_2O. [dinitro-BPTI] = 2.43 mM. Total La(III) + Pr(III) = 17.8 mM with the indicated mole fraction of metal as Pr(III).

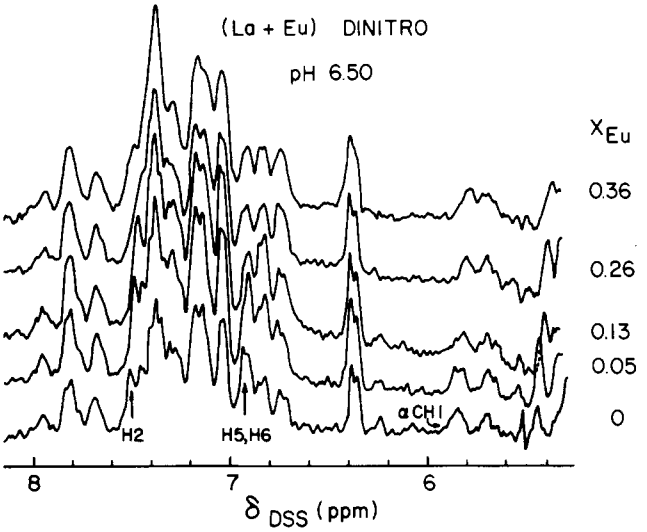

FIGURE 4: Eu(III) titration of dinitro-BPTI. Expanded presentation of the aromatic region showing shifts of nitrotyrosine 21 and α-CH1 protons in greater detail. Conditions are as described in Figure 4, except total lanthanide La(III) + Eu(III) is slightly higher.

is well determined. The metal-bound species is very similar to the free anion in terms of electronic structure, as evidenced by the very similar size and shape of the visible absorption. This indicates that the bonding to the metal is largely electrostatic, with a minimum of covalent interactions. This agrees with the general pattern of lanthanide complexes (Moeller, 1973) and is consistent with the observation that the lanthanide-induced shifts of ANTE have small or negligible contact contributions (Marinetti et al., 1975).

Lanthanide-Induced Shifts

Shifts and line broadening induced by lanthanide metals in the NMR spectra of BPTI and nitrated derivatives have previously been reported. Gd(III)-induced line broadening was used to obtain the distances of some resolved backbone NH and α-CH protons from the metal binding site at nitrotyrosine 21. These resonances also experienced shifts with Pr(III) and Eu(III). To quantitatively analyze the shift data for these resonances, whose protons have an unknown geometric relationship relative to the metal, it is necessary to calibrate the protein-lanthanide magnetic interactions. The induced shift behavior of the nitrotyrosine ring protons can be used for this calibration since they are at known positions relative to the metal. These shifts were obtained by lanthanide titration experiments in which the mole fraction of Pr(III) or Eu(III) was varied while the total lanthanide concentration was constant. La(III) is diamagnetic and cannot induce paramagnetic shifts but serves to buffer the concentrations of metal-bound species. Changes in the spectra with increasing mole fraction paramagnetic metal are due to the replacement of an increasing fraction of the La(III) complexes by Pr(III) or Eu(III) complexes. Examples of the spectra obtained are given in Figures 3 and 4 for Pr(III) and Eu(III), respectively. These spectra, taken from the low end of the titration with paramagnetic lanthanide, were used to quantitate the shifts of the ring protons. Using the reported assignments and the known pH dependence of the nitrotyrosine chemical shifts (Snyder et al., 1975), the lower trace of Figure 3 shows the H2 resonance of nitrotyrosine 10 at 8.22 ppm which shifts slightly downfield under the influence of Pr(III). The slightly broader singlet at 7.52 ppm, the H2 resonance of nitrotyrosine 21, shifts downfield by a considerably larger amount. In the upper traces, this resonance overlaps a phenylalanine resonance at 7.7 ppm. In

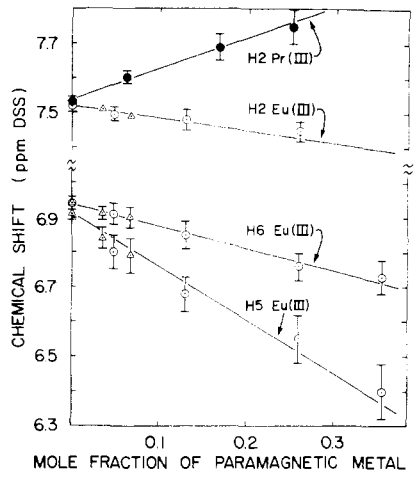

FIGURE 5: Observed nitrotyrosine 21 ring proton chemical shifts from the spectra of Figures 3 and 4. (⊙) Data at 250 MHz; (▲) data at 270 MHz. The points were obtained by examination of expanded overlays of sequential spectra, or by direct difference spectra. The lines are least-squares fits.

the absence of Pr(III), the H5 and H6 resonances of nitrotyrosine 21 are superimposed at 6.94 ppm and overlap one of the three doublets of area one of tyrosine 35. The positions of the H-5 and H-6 resonances could not be determined in the presence of Pr(III). The figure also shows the resolved backbone NH resonances in the region from 8 to 10.8 ppm and the α-CH1 resonance at 5.82 ppm.[2] These latter shift only by a small amount due to the relatively low concentrations of paramagnetic metal employed here, in contrast to the previously reported spectra at high concentrations (Marinetti et al., 1976). Figure 4 shows an analogous series with Eu(III) expanded to show the aromatic region in more detail. Note particularly the upfield shift of the nitrotyrosine 21 H5 and H6 resonances, exposing the three upfield tyrosine 35 doublets, and the movement of the H2 resonance of nitrotyrosine 21. These nitrotyrosine resonances become particularly difficult to observe as the mole fraction of paramagnetic metal is increased because of the concomitant chemical exchange line broadening, which is increased at the high magnetic fields used to obtain resolvable protein spectra. These data and similar titrations at 270 MHz using difference spectroscopy in some cases are summarized in Figure 5. Due to the chemical exchange line broadening cited above, the points above X_{Eu} = 0.2 are only estimates. The identification of the faster shifting component with H5 and the slower component with H6 is not a rigorous assignment and will be discussed below. The spectra were not taken at lower or higher pH's where assignable H5 and H6 resonances start out resolved because at lower pH the binding of metal is sharply decreased, and at higher pH the lanthanides are insoluble. Using the experimental stability constants, and correcting for BPTI's carboxyl groups by assuming 40% saturation at the pH of the lanthanide experiments here, the slopes of the lines in Figure 5 can be used to calculate bound shifts for the nitrotyrosine 21 ring protons. The results of the calculations are summarized in Table II. The slopes were obtained by a linear regression analysis weighting the data in accordance with their experimental error, which is indicated by the bars in Figure 5. The results for ANTE are given for comparison. It can be seen that the shifts with both Pr(III) and Eu(III) are consistently lower for nitrotyrosine 21 compared with ANTE. The large uncertainties in the bound shifts arise from the cumulative effects of errors both in the slopes of Figure 5 and the errors in the stability constants used in the calculations.

[2] The numbering of the NH (1 through 9) and α-CH (1) resonances correspond to the order of appearance in decreasing chemical shift from internal DSS in native BPTI at pH 7. In contrast, particular NH and α-CH protons (*19* through *22* and *31* through *33*) are numbered according to their position in the polypeptide sequence and appear in italics.

TABLE II: Nitrotyrosine 21 Ring Proton Shifts.

Proton	Metal	Slope[a]	Bound Shift[b]	ANTE[c]
H2	Pr	0.9 ± 0.3	1.9 ± 0.8	4.0
H2	Eu	−0.4 ± 0.1	−0.8 ± 0.4	−1.3
H5[d]	Eu	−1.7 ± 0.4	−3.6 ± 1.4	−7.4
H6[d]	Eu	−0.7 ± 0.3	−1.4 ± 0.7	−4.0

[a] From linear regression of data of Figure 5 in ppm. A downfield shift is positive. [b] Calculated shift relative to the species with La(III) bound using data of Figure 5 and Table I, in ppm. [c] Observed bound shifts in *N*-acetyl-3-nitrotyrosine ethyl ester in ppm (±10%) (Marinetti et al., 1975). [d] Assuming the fast moving component of Figure 5 is H5. See text.

Analysis

Nitrotyrosine chelation of Gd(III) in dinitro BPTI was used to obtain the distances of resolved backbone protons from the metal binding site nitrotyrosine 21 (Marinetti et al., 1976). The Gd(III)-induced line broadening localizes a given nucleus within a spherical shell in space but the direction of the metal–nucleus vector is not determined. Information concerning the orientation of this vector is contained in the lanthanide-induced shifts, which for protons arise mostly from the through-space dipolar interaction. These shifts are described by a function of the form (Bleaney, 1972):

$$(\Delta\omega/\omega_0) = r^{-3}[K_0(3\cos^2\theta' - 1) + K_2(\sin^2\theta'\cos 2\phi')] \quad (1)$$

where ω_0 is the Larmor frequency, K_0 and K_2 are constants related to the elements of the magnetic susceptibility tensor, χ, (r, θ', ϕ') are the polar coordinates of the nucleus relative to the metal expressed in the principal axis system of the metal complex, in which the χ tensor is diagonal. The orientation of this special coordinate system in the molecule is not known a priori. To calculate the shifts, eq 1 must be reexpressed, most conveniently done by expressing the trigonometric functions in terms of the normalized spherical harmonics (Marinetti et al., 1975) whose properties are well known (Brink and Satchler, 1968). The result is:

$$(\Delta\omega/\omega_0) = r^{-3}\sum_{m=-2}^{2} B_m C_{2m}(\theta, \phi) \quad (2)$$

where (r, θ, ϕ) are the polar coordinates of a point in an arbitrary coordinate system, C_{2m} is a normalized spherical harmonic, and B_m is a complex coefficient. The shift must be a real number, so $B_{-m} = (-1)^m B_m^*$. The B_m coefficients are related to the elements of the magnetic susceptibility tensor expressed in the arbitrary coordinates, but operationally they can be regarded as unknown parameters which are determined by experiment. There are five independent parameters, B_0 (which is pure real), and the real and imaginary parts of B_1 and B_2; so, in general, at least five experimental shifts are necessary to determine the magnetic parameters for a given metal. This number can be reduced if there is any symmetry present in the

complex, whether inherent or acquired as a result of rapid averaging of conformational isomers. In the case of an n-fold axis of symmetry, taking the n-fold axis as the z axis of the coordinate system, it can be shown that $B_1 = 0$ for a twofold axis, and both B_1 and $B_2 = 0$ for threefold or higher (Briggs et al., 1972). For the case of a reflection plane, choosing the z axis normal to the plane, symmetry requires $B_1 = 0$. Reexpressing eq 2 for this gives:

$$(\Delta\omega/\omega_0) = r^{-3}[A_0(3\cos^2\theta - 1) + A_2\sin^2\theta\cos 2(\phi - \eta_2)] \quad (3)$$

where the three parameters A_0, A_2, and η_2 are now real.

There are really two types of symmetry possible. First there can be symmetry present in the magnetic susceptibility tensor itself, due to a symmetric arrangement of the atoms in the coordination sphere, or averaging over isomers or both. The second type is due to the symmetric placement of the observed nuclei. For example, the ring plane of nitrotyrosine is clearly a symmetry plane for tne ring protons. It can also be a symmetry plane for the $\beta\beta'$ and α hydrogens, due to conformational averaging about the C_β–C_γ and C_α–C_β bonds which generates a reflected image point through the plane for each conformer position. If these conformers are about equally populated, this results in effective planar symmetry since the asymmetric component of one conformer is cancelled by that of its reflected image. The difference is that symmetry of the first type *imposes* the simplifications in the shift expression for all points in space, whereas the second type *appears* to fit the simplified form, but this would only be true for particular nuclei. This has important consequences for the use of shift probes in proteins since the side chains of a protein near a metal binding site are unlikely to have sufficient motional freedom to result in symmetry of the second kind. Thus, type two symmetry present in a model compound could prevent measurement of all five parameters which would be needed if the protein bound chelator was fully asymmetric.

In model compound work with ANTE (Marinetti et al., 1975) it was found that only three independent parameters were necessary to fit the experimental shifts for the H2, H5, H6, $\beta\beta'$, and α protons regardless of the coordinate system chosen for the calculation. This is a consequence of type two symmetry caused by internal motion as discussed above, and therefore it is not possible to test experimentally for the presence of type one symmetry. However, consideration of the geometrical isomers possible for the most likely metal coordination polyhedra (nine coordinate trigonal prisms, eight coordinate square prisms and antiprisms) shows that the ring plane is expected to be an effective symmetry plane, assuming the exchange between isomers is fast on the NMR time scale. Therefore, in analyzing the lanthanide-induced shifts of BPTI protons, the expression for type one symmetry (eq 3) will be used.

One final point concerning the use of the shift probes is that a given shift is not associated with a unique position in space. In fact there are eight points in space which have identical shifts, regardless of the presence of any symmetry. This is most easily seen by reference to eq 1. In the principal axis system, the points $(r, \pm\theta', \phi')$, $(r, \pm\theta', -\phi')$, $(r, \pm\theta', \phi' + \pi)$ and $(r, \pm\theta', \pi - \phi')$ all yield the same calculated shift. There is a unique shift for any point (r, θ, ϕ) so that given the coordinates of the nuclei shifts may be calculated and compared with the experimental values.

For practical reasons the comparison between observed and calculated shifts was undertaken for the Pr(III) data. The shifts induced by Eu(III) were smaller and only the nitrotyrosine ring protons and the α-CH1 resonances shifted by significant amounts. The analysis of the Pr(III) data requires that the three parameters of eq 3 be evaluated for Pr(III) bound to nitrotyrosine 21. Only the H2 ring proton was observable in the Pr(III) experiments, so a direct experimental measurement could not be done. It was assumed that the parameters describing the Pr(III)-induced shifts of nitrotyrosine 21 could be obtained simply by scaling the ANTE parameters to agree with the observed shift for the nitrotyrosine 21 H2 resonance. Some justification for this can be found in the Eu(III) shifts for the ring protons (see below). The coordinates used for calculated shifts were obtained using the known x-ray structure of BPTI (Diesenhofer and Steigemann, 1974) and standard geometries. Only the backbone positions consistent with the Gd(III)-induced relaxation (NH's and α-CH's *19, 20, 21, 22, 31, 32,* and *33*) were used in the calculations.

TABLE III: Magnetic Parameters.[a]

Data Set	A_0	A_2	η_2
Pr-ANTE[b]	−592	−1583	−1.07
Eu-ANTE[b]	−411	1690	−1.20
Eu-21 H5-fast[c]	−150	291	−1.00
Eu-21 H5-slow[c]	−1670	3070	−1.31
Pr-21-H5-fast[d]	−277	−740	−1.07

[a] Parameters describing dipolar shift of eq 3 using a coordinate system with z normal to the ring plane, x bisecting the O-M-O triangle of the bidentate function of the nitrotyrosine, and the ring oriented with H2 in quadrant IV; H5 and H6 are in quadrant I. A_0 and A_2 are ppm Å^3; η_2 is in radians. [b] Data from model compound (Marinetti et al., 1975). [c] Data from this paper for the two possible assignments of the fast and slow components of Figure 5. [d] Estimates based on scaling of Pr-ANTE parameters (see text).

Table III shows the results of analysis of the different data sets using eq 3. There is some ambiguity in the assignment of the fast shifting component of Figure 5 to the H5 or H6 proton of nitrotyrosine 21. This was resolved by consideration of the data of Figure 4 for the α-CH 1 resonance, which gave a slope of -0.18 ± 0.11 ppm when analyzed in the manner used for the ring protons. Using the parameters in Table III and the coordinates noted above, slopes were calculated for the backbone α-CH's. Only the parameters with the fast component assigned to H5 gave calculated slopes which were in agreement with the observed value. The results using this assignment are compared with the ANTE results in Table II. The ratios of the shifts are H2:H5:H6 = 1:4.8 ± 2.2:1.8 ± 1 for nitrotyrosine 21 and 1:5.6 ± 0.8:3.0 ± 0.5 for ANTE. The ring protons of nitrotyrosine 21 behave qualitatively like those of ANTE but quantitative differences are present in the magnitude of the induced shifts. The parameters for Pr(III) ANTE were scaled to give the "Pr(III) nitrotyrosine 21" parameters in Table II. The nitrotyrosine ring may have two orientations corresponding to 180° rotation about the C_β–C_γ bond. Shifts were calculated for the NH and α-CH protons for both orientations of the nitrotyrosine ring in the protein as well as weighted averages of the two orientations. Examination of the x-ray structure of BPTI reveals that the orientation with the nitro group at carbon $\epsilon 2$ (orientation $\epsilon 2$) has the nitro group buried in the protein and therefore may present steric barriers to a hydrated lanthanide ion. Thus, the most reasonable situatior is that the shift will be a weighted average of orientations $\epsilon 1$ and $\epsilon 2$ with the fraction (α) of $\epsilon 1$ lying between 50 and 100%. The results are given schematically in Figure 6 which presents

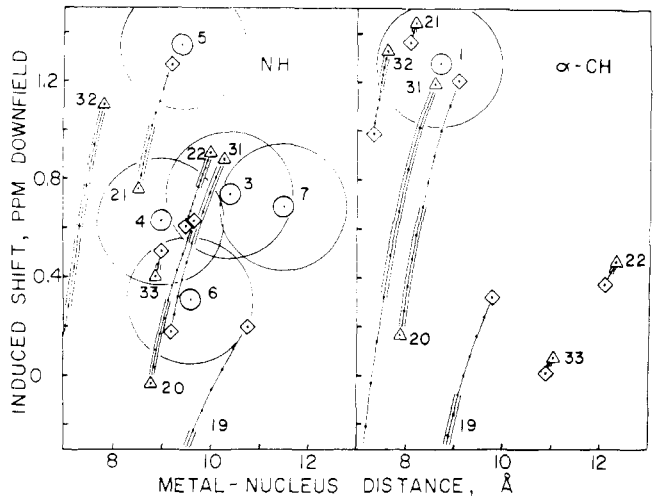

FIGURE 6: Summary of shift and distance calculations for backbone NH and α-CH resonances. The calculated shifts and distances for positions *19, 20, 21, 22, 31, 32,* and *33* are plotted for various averages of the two orientations of the nitrotyrosine 21 ring. The curves go from 100% ε1 (▲) to 100% ε2 (⊡) in increments of 10%. The triply lined regions (corresponding to 50 to 100% ε1) are the most likely. The observed data are plotted as circles with the large circle representing the estimated error. The best assignment for a given resonance is the curve which comes closest to it.

the simultaneous variation of the calculated shift and distance for the NH and α-CH protons with orientational averaging of nitrotyrosine 21. Each backbone position generates a curve in the distance–shift plane, with the most likely region (50 to 100% ε1) corresponding to the thickened region of the curve nearest the triangle end. The data points are indicated as circles with their estimated error indicated by the large circle surrounding the point. The best choice of backbone position for a given resonance is the curve which is closest to it. The best choices are listed in Table IV for three cases of metal binding at orientation ε2: (a) strongly hindered ($\alpha = 1$); (b) intermediate ($\alpha = 0.75$); and (c) weakly hindered ($\alpha = 0.5$).

TABLE IV: Summary of Resonance Assignments from Lanthanide Data.[a]

Obsd Resonance	Backbone Position: Case A Strongly Hindered $\alpha = 1$	Case B Intermediate $\alpha = 0.75$	Case C Weakly Hindered $\alpha = 0.5$
NH5	32	21	21
NH3, NH7	22, 31	22, 31	22, 31
NH4	21	33	33
NH6	33	20	20
αCH1	31, 21, ~32	21, ~32	21

[a] Best fit assignments for three cases of orientational averaging from the data contained in Figure 6. See text.

Discussion

Lanthanide ions bound to the nitrotyrosine ligands of dinitrated derivatives of BPTI induce line broadening and shifts in several of the resolved NH and α-CH resonances. The analysis of the Gd(III)-induced line broadening (Marinetti et al., 1976) was relatively straightforward and allowed calculation of distances between the broadened nuclei and the protein bound Gd(III). In conjunction with the known x-ray structure of the protein (Diesenhofer and Steigemann, 1974), this allowed an assignment of the affected NH protons to a small group of positions on the polypeptide backbone. However, the analysis of the Pr(III)- and Eu(III)-induced shifts for determination of the angular placement of the shifted nuclei has been more involved. Because several assumptions were required to carry the analysis of the obtainable experimental data to completion, the resulting assignments are somewhat ambiguous. However, they can be tested against other independent evidence for consistency as will be shown below. One use of the tentative BPTI assignments is as a guide for the planning and interpretation of other experiments such as decoupling and intramolecular nuclear Overhauser enhancements between the NH and α-CH resonances (Rowan and Markley, unpublished results). More generally the detailed analysis presented above illustrates the difficulties inherent in the lanthanide shift experiment and permits an evaluation of the requirements which must be met in the application of the lanthanide induced shifts to structural studies in proteins.

The assumptions made in the analysis, based in part on the experience with the model compound, are the following: (1) that the magnetic susceptibility tensor has an effective plane of symmetry; (2) that the Pr(III)-induced shifts of the three nitrotyrosine ring protons H2, H5, and H6 behave qualitatively similar to the model compound and scale down in magnitude, as observed for the Eu(III)-induced shifts; and (3) that the nitrotyrosine ring H5 proton undergoes the largest shift (as in the model compound). Without these assumptions, one would in general have to observe shifts for five nuclei at known symmetry unrelated positions, and this is not possible for the Pr(III)-induced shifts in dinitro-BPTI. Several observations can be made concerning the results obtained using the above assumptions. First, and most important, the shifts calculated are quite reasonable: there is good complementarity between the positions selected by the Gd(III)-induced line broadening and the Pr(III)-induced shifts. The general agreement suggests that the approximations made in the analysis are reasonable. Second, despite the ambiguities due to orientational effects, the pair of NH resonances 3 and 7 are consistently assigned to positions *22* and *31*. Examination of a space-filling model of BPTI shows position *22* to be very buried, consistent with the observation that NH3 is the resonance most resistant to exchange with solvent D_2O (Masson and Wüthrich, 1973; Karplus et al., 1973). Third, the results are consistent with the NMR coupling constant data. Calculation of the dihedral angles from the x-ray structure shows that positions *19* and *32* have small dihedral angles (140–145°), *33* is intermediate (150–155°), and the rest are large (165–180°). Coupling constants were calculated using a modified Karplus relation and the above angles (Barfield and Karplus, 1969; James, 1975) and compared with the apparent coupling constants reported for the NH resonances of BPTI (Karplus et al., 1973). Qualitatively, the assignments of Table IV (cases B and C) are in agreement with the predicted values since the NH's assigned to positions with small and large dihedral angles have small and large coupling constants, respectively. There are differences in absolute magnitude (the observed values are lower than the predicted by 1 to 2 Hz) but considering the possible errors in the choice of parameters in the calculation and also the large natural line widths of the NH resonances (for which the apparent coupling constant will be less than the true value),

these differences are not excessive. Fourth, in the case of the α-CH1 resonance the x-ray structure reveals that both the α-CH *21* and *31* protons lie near the plane of the nitrotyrosine 21 ring. This is consistent with the downfield shift of the α-CH1 resonance even in the absence of the metal ion, both in native and nitrated proteins. An indirect ring current effect for *31* along with possible covalent effects for *21* could explain the observed pH dependence of the chemical shift of the α-CH1 resonance, which titrates with a proton stability constant equal to that of nitrotyrosine in dinitro-BPTI and equal to tyrosine in native BPTI. The latter is clearly seen in published 360-MHz spectra (Wagner and Wüthrich, 1975) where the α-CH1 resonance is resolved even in native BPTI due to the high magnetic field. Finally, analysis of the x-ray structure (Diesenhofer and Steigemann, 1974) shows NH's *20, 21, 22, 31,* and *33* are involved in hydrogen bonds in a section of β sheet which is surrounded by aromatic side chains. This is where one would expect nonexchanging NH's to be found, consistent with the lanthanide NMR assignments.

The analysis and results presented above illustrate both the practical requirements for and the information available from the lanthanide shift experiments. The additional structure information contained in the shifts, as compared with the line broadening induced by Gd(III), is quantitatively interpretable but more parameters must be determined. In general, the shifts will require that resonances for five nuclei at a known position relative to the metal be observed, although this can be reduced to three or less if symmetry is present. In the line-broadening experiments, only one such resonance must be observed to calibrate the Gd(III)-induced relaxation. Due to the simpler form of the interaction, the Gd(III) distance experiment is the easiest to perform and interpret and would certainly be the starting point for any lanthanide NMR experiments. The shift data have been used here as an additional aid in assigning some of the resolved backbone NH resonances of a protein. The ultimate goal is to use the assigned resonances as structural probes of the molecule in solution. The x-ray structure provides a guideline for assignments and is the starting point in any investigation of structure. NMR can extend such studies by monitoring the changes in assigned resonances induced by external effects such as the addition of substrates, inhibitors or effectors to an enzyme or changes in solvent conditions causing denaturation of a protein. The lanthanide-induced effects on the NMR spectrum can be used to quantify such structural changes in solution, particularly changes in distance using Gd(III) as a relaxation probe.

The nitrotyrosine chelator has a very distinct advantage relative to the use of naturally occurring lanthanide binding sites since it offers three ring protons at a known geometrical position relative to the metal to serve as calibration points for the magnetic dipolar interaction. Comparison of the magnetic parameters in Table III shows calibration to be necessary since the nitrotyrosine 21 parameters differ from those of ANTE, presumably caused by the effect of neighboring charges on the protein on the electrostatic potential at the lanthanide metal ion. Use of the ANTE parameters would have led to calculated shifts which were too large. Other favorable aspects of this probe such as its being a known locus in the structure, and existence of fast exchange between free and metal bound nitrotyrosine ligands have been discussed in regard to the Gd(III) work (Marinetti et al., 1976). Our results indicate that the lanthanide nitrotyrosine probe particularly Gd(III) should provide a useful tool for NMR investigations of protein structure.

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