pected for H-28. Similarly, "oogoniol" was resolved into two components, presumably **2** (R = H) and **3** (R = H), but complete characterization was not possible because of insufficient material.

Biological activity of **2** (R = $(CH_3)_2CHCO$) and **3** (R = $(CH_3)_2CHCO$) was determined by the method of Barksdale and Lasure.[20] Addition of a dilute solution of the compound in MeOH–water (proportion of MeOH, 1% or less) to a culture of the female strain *Achlya ambisexualis* 734 caused the formation of oogonial initials. These became visible after about 12 h and reached a maximum number in 24–48 h. Solutions of **3** were active at a concentration as low as 50 ng/mL but a higher concentration of **2** (50 μg/mL) was required to induce formation of oogonial initials.

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# Paramagnetic Ion Induced Perturbations in the $^1H$ NMR Spectrum of Lysozyme: A Reassignment of the Tryptophan Indole NH Resonances[1a]

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**Abstract:** In $H_2O$ the 360-MHz $^1H$ NMR spectrum of the egg white lysozyme, an enzyme which has six tryptophan residues, contains only five resolvable indole NH resonances. These peaks have been assigned to specific tryptophan residues on the basis of the shift perturbations induced by $Co^{2+}$ and line broadenings induced by $Gd^{3+}$. Since each of these perturbations obeys a different geometric relationship, the agreement of the two sets of assignments provides a check of the overall method. The results of inhibitor binding studies, chemical modification experiments, and deuterium isotope exchange rates are discussed in terms of the new assignments. Apart from any specific conclusions reached on HEW lysozyme it is clear that for many macromolecules the line broadenings induced by $Gd^{3+}$ can be analyzed in terms of absolute metal proton distances. In making these assignments we have made use of the crystallographic data for HEW lysozyme. In general, the application of the methodology presented here is restricted to those biomolecules whose structure has been determined by an independent method.

The increased resolution and sensitivity available through the use of superconducting nuclear magnetic resonance spectrometers have made possible a wide variety of $^1H$ NMR investigations into the structure and dynamics of proteins in solution.[2] An essential first step in any such study is the assignment of a particular resonance in the complex $^1H$ NMR spectrum of the protein to a given hydrogen atom in the molecule. This process is usually accomplished in two steps. The first step is the assignment of the resonance to a particular kind of amino acid. The second is the identification of the particular peak with a specific residue in the sequence of the protein. Methods for making these assignments have been reviewed in the literature.[2-6] This communication describes an approach to the problem of making such assignments which is based on the stereospecificity of electron–nuclear interactions present in paramagnetic complexes.[7]

There is an extensive literature which deals with the applications of paramagnetic ion effects to the study of configurations and conformations of molecules in solution.[8] Strategies for employing these paramagnetic effects in conjunction with structural information available from X-ray crystallographic techniques to determine the solution structure of proteins have also been described.[5,6,9-11] Previous efforts in our laboratory have been directed at the development of a quantitative, statistically valid, computer-based method for the analysis of the chemical-shift perturbations and relaxation rate enhancements induced by paramagnetic metal ions.[9,12] The present study illustrates an application of this approach to the assignment of the tryptophan indole NH resonances of lysozyme.

Lysozyme is a low molecular weight enzyme (~14 700) with a known amino acid sequence.[13,14] The X-ray crystallographically determined structure for the native enzyme[15-17] as well as Fourier difference maps for various metal complexes[18,19] have been reported. This enzyme has been shown to bind polyvalent cations in solution[5,6,9,10,20-30] with side-chain carboxyl groups of Glu-35 and Asp-52 forming at least part of the metal binding site.[26] Although this enzyme contains six tryptophan residues at sequence positions 28, 62, 63, 108, 111,

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and 123, respectively, only five indole NH resonances have been observed in its NMR spectra.[29,31] Assignments of these five resonances based on a combination of data resulting from chemical modification studies, deuterium exchange kinetics, and inhibitor binding studies have been reported.[31] The latter studies are of particular interest since three of these tryptophans are at, or near, the active site of the enzyme. On the basis of $Gd^{3+}$-induced broadening, Campbell et al. have reassigned the most upfield of these five resonances to the tryptophan 108 NH proton,[29] casting doubt on the remaining assignments. We report a new set of assignments for these five tryptophans which can be made on the basis of shift perturbations observed with $Co^{2+}$ and line broadenings induced by $Gd^{3+}$. We have made use of the shifts induced by $Zn^{2+}$ and the line broadenings observed with $La^{3+}$ as diamagnetic corrections. We feel that these experiments will stand alone as a separate method of assignment.

## Experimental Section

Crystalline salt-free HEW lysozyme (three times recrystallized) was purchased from Worthington Biochemical Corporation (Freehold, N.J.). The protein was dissolved in $H_2O$ and its concentration determined spectrophotometrically.[32] Stock solutions of $CoCl_2$ and $ZnCl_2$ were prepared by dissolving the appropriate weight of these salts in the lysozyme solution. Stock solutions of $LaCl_3$ and $GdCl_3$ were prepared from the hydrates of those salts (Alfa-Ventron). Their concentrations were determined by a complexiometric titration with EDTA employing arsenazo as an indicator [33] By micropipetting aliquots of the stock solutions containing both metal ions and lysozyme into the lysozyme solution, the concentration of the metal was varied while the protein concentration was kept constant. Spectra were recorded on a modified Bruker HX-360 (Stanford) spectrometer operating in correlation mode.[34] Each spectrum was the sum of 100 scans. All spectra were recorded at a pH of 5.1 at 55 °C to minimize aggregation. The temperature was determined to ±1 °C from the chemical shifts of ethylene glycol Chemical shifts were measured relative to tetramethylammonium chloride as internal standard. Computations were performed on an IBM-370 computer.

## Theoretical Background

We briefly outline the theoretical basis of our approach to the data analysis and interpretation. The line broadenings (transverse relaxation rate enhancements) of a given resonance in the presence of a paramagnetic metal ion can be expressed as

$$1/T_{2p} = \pi(\Delta\nu_{1/2}{}^{M} - \Delta\nu_{1/2}{}^{0}) \quad (1)$$

where $\Delta\nu_{1/2}{}^{M}$ and $\Delta\nu_{1/2}{}^{0}$ are the line widths at half-height (in hertz) of the resonance with and without the paramagnetic metal ion and $1/T_{2p}$ is the paramagnetic contribution to the transverse relaxation rate in reciprocal seconds. Under certain conditions (i.e., the absence of any outer-sphere relaxation and $1/T_{2M} \geq \Delta\omega_M$ where $\Delta\omega_M$ is the chemical-shift difference between the free and bound states), the concentration dependence of $1/T_{2p}$ is given by[35,36]

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

where $P_M$ is the fraction of ligand bound to the metal ion, $1/T_{2M}$ is the relaxation rate of the resonance in its complexed state, and $\tau_m$ is the mean residence time of the ligand in its complexed state. When the relaxation enhancement arises solely through the dipolar interaction, the following equation holds:[37]

$$1/T_{2M} = \frac{\gamma_I{}^2\beta^2 S(S+1)g^2}{15r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I{}^2\tau^2} + \frac{13\tau_c}{1+\omega_S{}^2\tau_c{}^2}\right] \quad (3)$$

$\omega_I$ and $\omega_S$ are the nuclear and electronic Larmor frequencies related to the resonance frequency $\nu$ by $\omega_I = 2\pi\nu$ and $\omega_S = 660\ \omega_I$, $\gamma_I$ is the magnetogyric ratio of the nucleus being observed, $\beta$ is the Bohr magneton, $S$ is the total electron spin, and $r$ is the distance between the nucleus and the paramagnetic ion. The correlation time $\tau_c$ in the above equation is defined as

$$1/\tau_c = 1/\tau_s + 1/\tau_r + 1/\tau_m \quad (4)$$

where $\tau_r$ is the rotational correlation time of the complex, $\tau_s$ is the electron spin relaxation time of the paramagnetic electron, and $\tau_m$ is the mean residence time of the ligand in the metal complex. The frequency dependence of $\tau_s$ has been derived by Reuben and Luz[38]

$$1/\tau_s = C\tau_v/[1 + B(\omega_s{}^2\tau_v{}^2)] \quad (5)$$

where $\tau_v$ is the correlation time which reflects the rate at which collisions modulate the zero field splitting, $B$ is a constant related to the zero field splitting, and $C$ is a constant for the particular spin system in question. The temperature dependence of all of the correlation times obey a relationship of the form

$$\tau = \tau_0\, e^{E/RT} \quad (6)$$

where $\tau_0$ is the intrinsic correlation time, and $E$ is the energy of activation for the particular process.

## Chemical-Shift Perturbations

The total shift observed in the resonance of a given nucleus in the presence of paramagnetic ions can be expressed as

$$\Delta = \Delta_{CF} + \Delta_C + \Delta_D \quad (7)$$

where $\Delta_{CF}$ is the complex formation shift and is diamagnetic in origin, $\Delta_C$ is the shift arising from the Fermi contact interaction (a through-bond effect), and $\Delta_D$ is the shift arising from the dipolar or pseudocontact interaction (a through-space effect). The value of $\Delta_{CF}$ can usually be determined by measuring the shift observed upon complexation with a suitable diamagnetic metal.

In complexes with isotropic magnetic susceptibility tensors, the dipolar electronnuclear interactions are effectively averaged out. For complexes in which the susceptibility is anisotropic, the dipolar shift is given by[7]

$$\Delta_D = K_1(1 - 3\cos^2\theta)/r^3 + K_2(\sin^2\theta\cos 2\phi/r^3) \quad (8)$$

where $K_1$ and $K_2$ are constants related to elements of the magnetic susceptibility tensor of the metal ion, and $r$, $\theta$, and $\phi$ are the spherical polar coordinates of the nucleus relative to the principal magnetic axis system of the metal atom. If $K_2 \neq 0$, the shifts arise from a non-axially-symmetric magnetic susceptibility tensor; $K_2 = 0$ or $K_2 \ll K_1$ represents the axially symmetric case.

## Results and Discussion

**$Gd^{3+}$ Experiments. 1. Calculation of Relative Metal–Proton Distances.** The region of the lysozyme spectrum where the tryptophan indole NH resonances occur is shown in Figure 1a. On the addition of $La^{3+}$ the most pronounced change is the downfield shift of the E resonance. Note that the line widths of these peaks are unaffected by the presence of $La^{3+}$ ions. Figures 1d–g show the same region of the spectrum in the presence of various concentrations of $Gd^{3+}$ ions. As has been pointed out by Marinetti et al.,[39] the presence of $La^{3+}$ in these experiments serves to minimize any binding of the $Gd^{3+}$ to the glassware.[27] From the spectra in Figure 1, it is clear that $Gd^{3+}$ produces differential broadenings in the five NH resonances, with the E resonance being broadened beyond detection after the first additions of $Gd^{3+}$. A similar observation has been made by Campbell et al.[29] Using the values of the dissociation constants for both the $La^{3+}$ and $Gd^{3+}$ complexes of lysozyme[28,40] and the concentrations of all of the species present, we calculated the values of $P_m$ for each concentration of

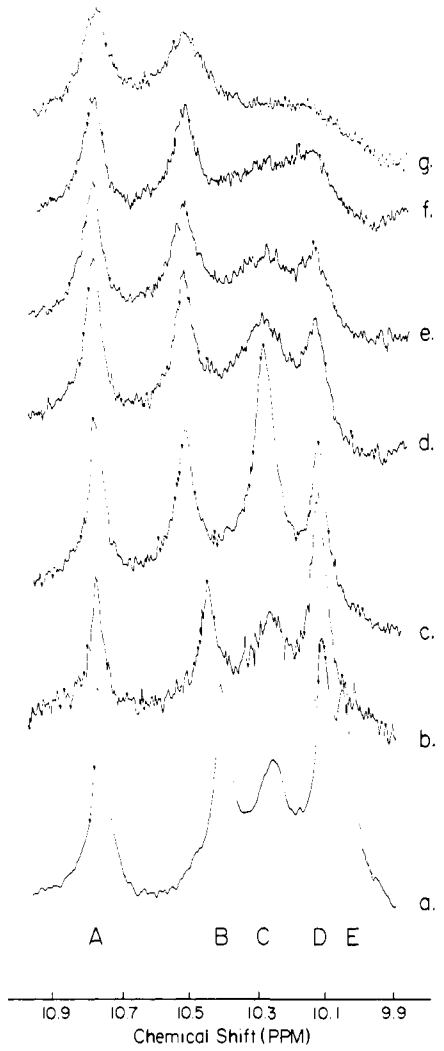


**Figure 1.** The tryptophan indole NH resonances of 6.32 mM HEW lysozyme (pH 5.1, 55 °C) in the presence of (a) no metal ions, (b) 2.2 mM $La^{3+}$, (c) 4.4 mM $La^{3+}$, (d) 4.4 mM $La^{3+}$ and 0.0006 mM $Gd^{3+}$, (e) 4.4 mM $La^{3+}$ and 0.13 mM $Gd^{3+}$, (f) 4.4 mM $La^{3+}$ and 0.20 mM $Gd^{3+}$, (g) 4.4 mM $La^{3+}$ and 0.26 mM $Gd^{3+}$.

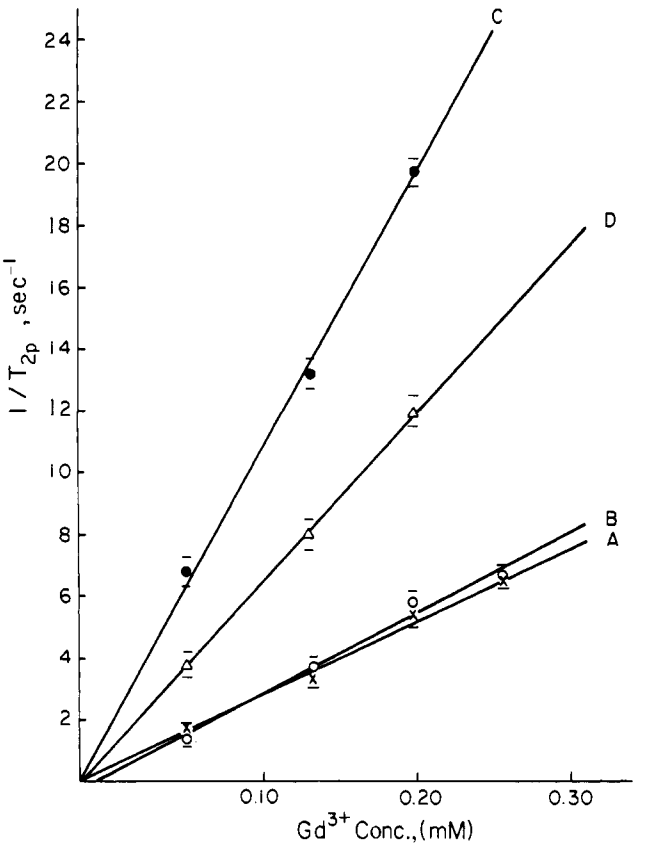


**Figure 2.** The variation in $1/T_{2p}$ (measured from the line width at half-height) of the resonances A, B, C, and D with $Gd^{3+}$ concentration.

$Gd^{3+}$.[41] The variations in the line widths at half-height of peaks A–D with gadolinium concentration are shown in Figure 2. The slopes of the lines in Figure 2 are related to the values of $1/T_{2M}$ (cf. eq 2) for each of the four indole NH hydrogens. The relative distance of each of these atoms to the $Gd^{3+}$ ion was computed by taking the inverse of the sixth root of the slopes. These distances, normalized to the value obtained for peak A are given in Table I. Using the coordinates for $Gd^{3+}$ obtained from either the crystal structure of the metal complex[19] or from an analysis of the NMR data of Williams et al.,[12] relative distances for the six tryptophan indole NH hydrogens were obtained. These distances are given in Tables II[42] and III. On the basis of a comparison of the observed and calculated relative distances, we have assigned resonances A–E to the indole NH protons of the tryptophans at sequence positions 123, 111, 63, 62, and 108, respectively.

**2. Calculation of Absolute Metal–Proton Distances.** In computing the absolute distances between the various tryptophan NH protons and $Gd^{3+}$, we employ an approach similar to the one described by Marinetti et al.[39] Generally the process of calculating distances using eq 3 involves specifying a value of $\tau_c$ for the molecule being studied. Finding a value for $\tau_c$ is, in general, nontrivial since $\tau_c$ is a combination of correlation times. Therefore the specification of $\tau_c$ in the present case requires estimates of $\tau_r$, $\tau_m$, and $\tau_s$ in eq 4. The discussion that follows has general implications on the feasibility of using eq 3 to calculate absolute metal–proton distances.

**Table I.** The Results of an Analysis of the $Gd^{3+}$-Induced Broadenings of the Five Tryptophan Indole NH Resonances Observed in the $^1H$ Spectrum of Lysozyme

| peak | $1/T_{2M}$,[a] $s^{-1}$ | rel distance, Å | absolute distance, Å |
|---|---|---|---|
| A | 168 ± 21 | 1.00 | 15.3 ± 0.3 |
| B | 187 ± 25 | 0.99 ± 0.03 | 15.1 ± 0.3 |
| C | 657 ± 122 | 0.80 ± 0.03 | 12.2 ± 0.4 |
| D | 390 ± 74 | 0.87 ± 0.03 | 13.3 ± 0.3 |
| E[b] | | | |

[a] Error limits were determined from an analysis of the results shown in Figure 2. [b] Broadened beyond detection after the first addition of $Gd^{3+}$.

The value of $\tau_s$ at 360 MHz and 55 °C can be obtained in two steps. A value of $\tau_v$ for the $Gd^{3+}$–lysozyme complex of 2.2 ± 0.4 × $10^{-11}$ s at 25 °C has been reported by Jones et al.[27] Using the value for $E_v$ of 6.0 ± 1 kcal/mol reported by the same authors in eq 6, we compute a value for $\tau_v$ of 8.0 ± 0.5 × $10^{-12}$ s at 55 °C. Using this value of $\tau_v$ together with the value of $B$ reported by Reuben and Luz[38] and the appropriate value of $C$, in eq 5, we obtain a value for $\tau_s$ of ~1.5 × $10^{-7}$ s at 360 MHz and 55 °C.

The exchange contribution, $\tau_m$, can be estimated from the stability constant and the rate constant for the forward binding process. As has been pointed out by Marinetti et al.,[39] this forward rate constant is nearly independent of the particular lanthanide ion for complex formation between the light lanthanides and oxygen-containing ligands. This conclusion was based on the kinetic data for lanthanide binding to oxalate[43]

**Table II.** Calculated Relative and Absolute $Gd^{3+}$ Proton Distance for the Tryptophan Indole NH Hydrogens Based on X-ray Crystallographic Data for the Triclinic Form of HEW Lysozyme

| atom | Cartesian coordinates,[a] Å | | | rel distance,[b] Å | absolute distance, Å |
|---|---|---|---|---|---|
| | *x* | *y* | *z* | | |
| Trp 28 NH | 2.3768 | 22.7252 | 22.3828 | 1.15 | 18.54 |
| Trp 62 NH | 5.1907 | 9.8541 | 38.6266 | 0.78 | 12.58 |
| Trp 63 NH | 2.3790 | 12.3388 | 34.1054 | 0.73 | 11.81 |
| Trp 108 NH | 5.7210 | 14.2716 | 27.5078 | 0.58 | 8.63 |
| Trp 111 NH | 9.7425 | 22.9124 | 24.9357 | 0.88 | 14.20 |
| Trp 123 NH | 13.7324 | 21.4752 | 16.3602 | 1.0 | 16.13 |
| $Gd^{3+}$ | 13.1078 | 9.9999 | 28.5098 | | |
| $Co^{2+}$ | 13.0497 | 9.8216 | 28.3159 | | |

[a] All hydrogen coordinates were calculated from the fractional crystal coordinate using an NH bond length of 0.9 Å assuming $sp^2$ hybridization. [b] Distances are reported relative to Trp 123 NH for ease of comparison with experimental results.

**Table III.** Calculated Relative and Absolute $Gd^{3+}$ Proton Distances and $Co^{2+}$-Induced Shifts for the Tryptophan Indole NH Hydrogens Based on X-ray Crystallographic Data For the Tetragonal Form of HEW Lysozyme

| atom | Cartesian coordinates, Å[a] | | | rel distance,[b] Å | absolute distance, Å | $Co^{2+}$-induced shift[c] |
|---|---|---|---|---|---|---|
| | *x* | *y* | *z* | | | |
| Trp 28 NH | −9.6831 | 25.0306 | 19.1939 | 1.17 | 18.4 | 0.11 |
| Trp 62 NH | 7.7466 | 23.6045 | 31.2562 | 0.92 | 14.4 | −0.30 |
| Trp 63 NH | 2.6757 | 22.2743 | 28.3617 | 0.82 | 12.8 | −0.66 |
| Trp 108 NH | 1.1001 | 22.1114 | 21.0816 | 0.54 | 8.4 | −0.43 |
| Trp 111 NH | −4.8846 | 30.7963 | 17.2685 | 0.99 | 15.6 | 0.32 |
| Trp 123 NH | −2.1952 | 30.5268 | 8.432 | 1.0 | 15.7 | 0.22 |
| $Gd^{3+}$ [d] | 8.4 ± 0.2 | 22.7 ± 0.1 | 16.9 ± 0.4 | | | |
| $Co^{2+}$ [e] | 7.8692 | 24.894 | 18.601 | | | |

[a] All hydrogen coordinates were calculated from the fractional, crystal coordinates using an NH bond distance of 0.9 Å assuming $sp^2$ hybridization. [b] Distances are reported relative to Trp 123 NH for ease of comparison with experimental results. [c] Shifts were calculated using the five parameters obtained in ref 9. [d] The gadolinium position is the one obtained in our previous analyses of the $Gd^{3+}$ induced broadenings reported by Williams et al.[12] [e] The $Co^{2+}$ position is the one inferred from the position reported by Jensen and co-workers for triclinic lysozyme (see ref 19 for details).

and murexide.[44] Since the ligands in lysozyme which bind $Gd^{3+}$ are very likely all oxygen containing (the carboxyl groups of Asp-52 and Glu-35), we can make use of the value for the forward ligation step reported in the literature for oxalate and murexide. In this way we calculate a value for $\tau_m$ of $1 \times 10^{-7}$ s using a value for the stability constant of $2 \times 10^3$ $M^{-1}$ [28,40] As will become evident shortly, although this estimate of $\tau_m$ may in fact be only an order of magnitude estimate, its precision is sufficient for our analysis. It is important to note that a comparison of $\tau_m$ and the values of $T_{2M}$ obtained experimentally clearly shows that $\tau_m$ can be neglected in eq 2.

A value for $\tau_r$ of $2.9 \times 10^{-9}$ s has been reported by Jones et al. for the $Gd^{3+}$–lysozyme complex at 25 °C.[27] Using the value of $E_r$ of 3 kcal/mol also reported by Jones et al. in eq 6, we obtain a value for $\tau_r$ of $1.9 \times 10^{-9}$ s at 55 °C. A comparison of the three correlation times needed for the computation of $\tau_c$ in eq 4 clearly demonstrates that $\tau_c$ is dominated by a contribution from the rotational correlation time. Let us examine the generality of this conclusion for other macromolecular complexes of $Gd^{3+}$. Our computations of $\tau_s$ are valid for most macromolecules since the frequency dependence of this correlation time ensures that at high spectrometer frequencies $\tau_s$ will become $\sim 1 \times 10^{-7}$ s. Since the exchange contribution, $\tau_m$, was estimated assuming a value for the rate constant for forward ligation which was essentially independent of the ligands, provided that they were oxygen containing, this part of our calculation is general. In addition, in order for $\tau_s$ to be negligible at the same level, it must be 20 times larger than $\tau_r$. Therefore our conclusions for lysozyme also apply to molecules that have values for $\tau_r$ of $2 \times 10^{-8}$ s or smaller with the appropriate stability constants for $Gd^{3+}$ binding such that $1/\tau_r$ is still 20 times larger than $1/\tau_m$. In using a single correlation time for lysozyme, we have assumed that neither anisotropic rotation nor internal motions are present. In cases where this assumption is not valid we suggest that the use of a single correlation time may still be adequate for the computation of absolute metal–proton distances. We note that, by taking the sixth root of the correlation function, a factor of two error in estimating the correct value of $\tau_r$ results in much smaller errors in the distance.

Since there are independent methods for measuring rotational correlation times of macromolecules (e.g., light scattering, depolarization of fluorescence, hydrodynamic properties, etc.), it should be possible, in the cases that meet the above criteria, to analyze $Gd^{3+}$-induced relaxation enhancements in terms of absolute $Gd^{3+}$–proton distances. We have calculated these various distances for lysozyme, using the appropriate values of the various parameters in eq 3. These distances are given in Table II. The corresponding distances obtained from X-ray crystallographic studies are given in Tables II and III. Assignments can be made by comparing the observed distances with the calculated distances in Tables II and III. From this analysis it is evident that the tryptophan resonance which is not observed in the $^1H$ NMR spectrum of lysozyme is at sequence position 28 and peaks A–E are Trp-123, Trp-111, Trp-63, Trp-62, and Trp-108, respectively. In the following sections we present an alternate method for making these assignments and a comparison of these results with the results of chemical modification experiments and inhibitor binding studies.

**$Co^{2+}$ Experiments. 1. Calculation of Expected $Co^{2+}$ Shifts.** In a previous analysis of the $Co^{2+}$-induced shifts of the high-field region of the $^1H$ NMR spectrum of HEW lysozyme, we fit the observed shifts to the five parameters necessary to de-

**Table IV.** The Limiting Shifts Induced by $Co^{2+}$ and $Zn^{2+}$ in Tryptophan Indole NH Resonances of the $^1H$ Spectrum of Lysozyme

| resonance | *I*, $Co^{2+}$-limiting shift,[a,b] ppm | II, $Zn^{2+}$-limiting shift,[a] ppm | I-II, corrected $Co^{2+}$-limiting shift,[a] ppm |
|---|---|---|---|
| A | 0.27 | 0.01 | 0.26 |
| B | 0.35 | 0.03 | 0.32 |
| C | −0.74 | 0.00 | −0.74 |
| D | −0.28 | 0.02 | −0.30 |
| E | −0.29 | 0.18 | −0.47 |

[a] All upfield shifts have a negative sign. [b] The association constant obtained for these data was 33 ± 5 $M^{-1}$ for $Co^{2+}$.

scribe the nonaxial form of eq 8 ($K_1$, $K_2$, and the three angles which describe the orientation of the magnetic susceptibility tensor of $Co^{2+}$ with respect to the crystal axis system).[9] Using these values of the five parameters, the coordinates of $Co^{2+}$ and the six tryptophan indole NH hydrogens, we calculated their six limiting shift values. These limiting shifts along with the Cartesian coordinates for the indole NH protons are given in Table III. Note that these calculations predict that the tryptophan NH's at sequence positions 62, 63, and 108 should shift upfield with tryptophan 63 being shifted the most. The other three indole NH resonances should shift downfield with Trp-28 being shifted the least.

**2. Binding Parameters from $Co^{2+}$ Shift Data.** We have assumed that $Co^{2+}$ forms a 1:1 complex with lysozyme. A similar assumption was made by McDonald and Phillips.[20] For such a complex the binding process can be written as

$$M + L \rightleftarrows ML \tag{9}$$

with an association constant, $K$, given by

$$K = [ML]/[M][L] \tag{10}$$

where M, L, and ML refer to metal, lysozyme, and metal-lysozyme complex, respectively, and brackets denote the equilibrium concentration. When the mean residence time of a ligand in a complex with a metal ion is much shorter than the the reciprocal of the chemical-shift difference between the resonance of the complexed and uncomplexed states, a signal which is a weighted average of the two states is observed. The resulting chemical-shift perturbations are given by

$$\delta^i_{obsd} = [ML]\Delta_i/L_T \tag{11}$$

where $\delta^i_{obsd}$ is the observed chemical shift of the $i$th nucleus, referenced to the chemical shift in the same nucleus with no metal present ($\delta^i_0$), $\Delta_i$ is its limiting chemical-shift value in the complexed state, also referenced to $\delta^i_0$, and $L_T$ refers to the total lysozyme concentration present. In general, fitting the shift perturbations of $i$ different resonances in the same molecule to eq 10 and 11 requires $i + 1$ parameters, $i$ values of $\Delta_i$, and a value for the stability constant.

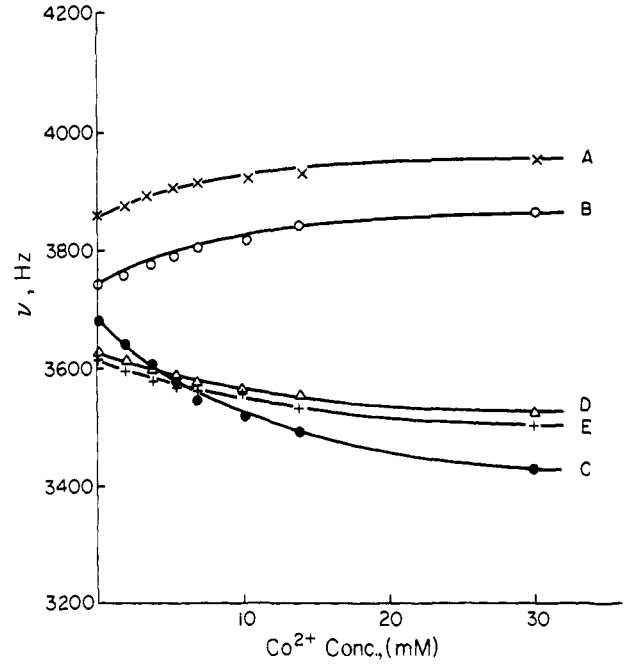


**Figure 3.** The variation in the chemical shift of the tryptophan indole NH resonances of HEW lysozyme with $Co^{2+}$ concentrations.

The addition of $Co^{2+}$ to a solution of lysozyme resulted in the perturbation of all five of the indole NH resonances. The variation in the chemical shift of each resonance with $Co^{2+}$ concentration is shown in Figure 3. Note that three of the peaks are shifted to higher field by $Co^{2+}$ and two are shifted to lower field. These data were analyzed using an algorithm which fit all of the data simultaneously to yield the six parameters necessary to describe the system (five $\Delta_i$ values and $K$). The chemical-shift perturbations of the same five resonances were monitored as a function of $Zn^{2+}$ concentration for the purposes of evaluating the $\Delta_{CF}$ term in eq 7. The results of both the $Co^{2+}$ and $Zn^{2+}$ analyses are presented in Table IV. A comparison of the $Co^{2+}$ experimental shifts in Table IV with the calculated shifts in Table III leads to the assignments of peaks A–E to the tryptophan indole NH's at sequence position 123, 111, 63, 62, and 108, respectively. Note that the assignments are identical with the ones reached on the basis of the $Gd^{3+}$ results.

**Comparison with the Previous Assignments.** The observations that formed the basis for the original assignments are summarized in Table V. In that study only three (A, B, and E) of the five tryptophan indole NH resonances in $D_2O$ were observed, indicating that the exchange rates of resonances C and D were fairly rapid. Estimates of the degree of solvent exposure

**Table V.** A Summary of the Results of Chemical Modifications, Inhibitor Binding Studies, and Deuterium Exchange Kinetics Data[a]

| resonance | assignment previous | assignment present | chemical modification Trp-62 oxindole | chemical modification Trp-108 oxindole | shifts obsd on inhibitor binding, ppm NAG | shifts obsd on inhibitor binding, ppm $(NAG)_2$ | deuterium exchange kinetics[b] free enzyme | deuterium exchange kinetics[b] +NAG |
|---|---|---|---|---|---|---|---|---|
| A | 111 (28) | 123 | | | 0 | 0 | 19.9 | no effect |
| B | 108 | 111 | | perturbed | 0 | 0 | 4.0 | decrease |
| C | 62 | 63 | perturbed | | −0.5 | −0.2 | too rapid to measure | decrease |
| D | 123 | 62 | perturbed | | 0 | 0 | too rapid to measure | remains too rapid to measure |
| E | 63 | 108 | | perturbed | 0.4 | 0.2 | ~22 | decrease |

[a] Reference 19. [b] Rate constants are reported in units of $10^{-3}$ $min^{-1}$.

of the six indole NH's made on the basis of X-ray data yielded the following order: Trp-28 ≪ Trp-108, Trp-111 ≪ Trp-63 < Trp-123 ≪ Trp-62. In a similar manner, it was predicted that the binding of inhibitor would decrease the exchange rates of the indole NH's of Trp-108, Trp-62, and Trp-63. It was found that the presence of inhibitor decreased the rates of exchange of peaks B, C, and E. In this way the group of peaks B, C, and E were identified as active site tryptophans. The binding of inhibitor brought about a shift in the resonance positions of peaks C and E, confirming the assignment of these two peaks to tryptophans at the active site.

The chemical modification of Trp-108 resulted in the perturbations of both peaks B and E. Because the exchange rate of peak B was found to decrease in the presence of inhibitor, it was assigned to Trp-108. The conversion of Trp-62 to an oxindole resulted in an apparent perturbation of peak C. It is, however, difficult to distinguish between a perturbation of peak C alone or perturbation in the resonance positions of peaks C and D. The most straightforward interpretation, which assigned peak C to the indole NH of Trp-62, was adopted in the previous study.[31] Peak A was assigned to the indole NH of either Trp-111 or Trp-28 because it was not an active site tryptophan and it showed the slowest exchange rate. Since peak E was perturbed on inhibitor binding and had a moderate exchange rate, it was assigned to Trp-63. This left peak D as the indole NH of Trp-123. This last assignment seemed reasonable in light of its rapid exchange rate and the fact that it was not perturbed on inhibitor binding. Thus, peaks A–E were assigned to Trp-111 (28), Trp-108, Trp-62, Trp-123, and Trp-63, respectively.

It is instructive to reexamine the above data in the context of assignments made on the basis of paramagnetic effects, since there must be consistency between the two sets of experiments. We have therefore compared each of our assignments with those of the previous studies.

**Peak E (Trp-108).** The resonance position of this peak is shifted on inhibitor binding. Its exchange rate is consistent with that predicted from the X-ray structure and the exchange rate was observed to decrease in the presence of inhibitor. In addition, the resonance position of this peak was perturbed upon oxidation of Trp-108. Note that this modification also perturbed peak B which we have assigned to Trp-111. It has been found that the oxidation of Trp-108 results in the formation of an ester bond between this residue and Glu-35.[45] The results of X-ray crystallographic studies suggest that, in the crystal, the chemical modification of Trp-108 results in only small movements of Glu-35 and Trp-108.[46] In contrast, NMR studies of this modified form of lysozyme show that the resonances of residues such as Tyr-23 and Ile-17 as well as Met-105 and Ile-98 are perturbed.[5] Since some of these residues are as far as 15 Å from Trp-108, it has been suggested that in solution the structural perturbations associated with the modification of Trp-108 are significantly larger than in the crystal.[5] Therefore, in solution, it is not surprising to find a perturbation in the resonance position of another tryptophan which is only three residues removed in the primary sequence from the site of chemical modification.

**Peak D (Trp-62).** In light of the previous discussion, it is reasonable to anticipate that the formation of an oxindole of Trp-62 might bring about perturbations in the resonance positions of the indole NH's of both Trp-62 and Trp-63. We therefore suggest that the less straightforward interpretation of the results of this chemical modification is correct. Note that peak D has a rapid exchange rate which remains too rapid to measure even in the presence of inhibitor. These observations are consistent with the predictions made on the basis of the X-ray structure. There is an inconsistency however, in the insensitivity of the chemical shift of this resonance to the binding of inhibitor since Trp-62 is thought to form a hydrogen bond with the inhibitor. Examination of the model of the active site of triclinic lysozyme in the presence of inhibitor reported by Jensen and co-workers[19] indicates that Trp-62 is further from the inhibitor than Trp-63. Therefore, we suggest that, in solution, the hydrogen bond proposed on the basis of the crystal structure[17] may not be present.

**Peak C (Trp-63).** Our interpretation of the results of chemical modification of Trp-62 is consistent with the assignment of peak C to Trp-63. This peak experiences an upfield shift on inhibitor binding, and has a rapid exchange rate which decreases in the presence of inhibitor. All of these observations are consistent with the predictions based on the X-ray structure which indicates the formation of a hydrogen bond between the indole NH of Trp-63 and an oxygen atom in the inhibitor molecule.

**Peak B (Trp-111).** We have previously accounted for the perturbations observed in peak B on oxidation of Trp-108. The fact that this peak has an exchange rate which is relatively slow is consistent with the predictions of solvent exposure expected for Trp-111. Although the rate of exchange of this peak is decreased in the presence of inhibitor, there is no significant change observed in its resonance position on the binding of inhibitor. These observations suggest that the presence of an inhibitor in the active site cleft of the enzyme may block access of solvent to the indole NH of Trp-111.

**Peak A (Trp-123).** We have assigned peak A to the indole NH of Trp-123. There are no significant perturbations observed in either the exchange rate or chemical shift of this peak on the binding of an inhibitor molecule. However, the exchange rate predicted for this tryptophan is much faster than was actually observed. This discrepancy may be accounted for by recognizing the fact that Trp-123 is at, or near, the surface of the enzyme and thus is likely to participate in interactions leading to the packing of the crystal. In solution, its environment may be quite different from the one found in the solid state. We suggest that the discrepancy between the observed exchange rate and the predicted extent of solvent exposure is a reflection of the variations in structure around Trp-123 in the two phases.

## Conclusions

The previous sections have demonstrated an application of paramagnetic ion effects to the assignment of specific resonances in the NMR spectrum of lysozyme. Part of the methodology necessary for making these assignments has been presented in this report. Other portions have been described in our analysis of $Gd^{3+}$-induced line broadenings[12] and $Co^{2+}$-induced chemical-shift perturbations in HEW lysozyme.[9] Since each kind of perturbation obeys a different geometric relationship, the consistency of the results of the analysis of each kind of perturbation provides a check of the overall method. In making these assignments we have made use of the crystallographic data for HEW lysozyme. In general, the application of the methodology presented here is restricted to those biomolecules whose structure has been determined by an independent method.

In addition to the specific conclusions reached on HEW lysozyme, we wish to emphasize the more general conclusion that can now be drawn regarding the use of $Gd^{3+}$ as a "relaxation reagent" in macromolecules. From our results, it is clear that, for $Gd^{3+}$–macromolecular complexes at high magnetic fields (~360 MHz), the dominant correlation time is probably the rotational correlation time of the complex. We have outlined some simple criteria for evaluating the feasibility of analyzing the $Gd^{3+}$-induced relaxation enhancements in such systems in terms of absolute metal–proton distances. As has been pointed out by others, the inverse sixth power distance relationship described in eq 3 for such interactions permits the calculation of fairly precise distances from relaxation data

which may contain rather large errors. Since the rotational correlation time of macromolecules can be measured independently by a variety of physical methods, we suggest that an analysis of $Gd^{3+}$ relaxation data in similar systems may be made in terms of absolute rather than relative distances following an approach similar to the one outlined in this report.[47]

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## References and Notes

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