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An Analysis of the Co^{2+}-Induced Nuclear Magnetic Resonance Perturbations of Hen Egg White Lysozyme†

R. E. Lenkinski,* D. G. Agresti, D. M. Chen, and J. D. Glickson

ABSTRACT: A general methodology is presented for analyzing dipolar shifts induced by paramagnetic ions in the nuclear magnetic resonance (NMR) spectra of ligand molecules. The method is applied to the shift perturbations induced by Co^{2+} in the spectrum of hen egg white lysozyme. A hypothesis testing scheme is employed to evaluate statistically the relative precision with which the axially symmetric and non-axially symmetric forms of the dipolar shift equation fit the observed data. The assumption of axial symmetry for the magnetic susceptibility tensor of Co^{2+} is rejected at the confidence level of 99%. Since the results presented here are similar to those reached in our analysis of lanthanide-induced shifts, we suggest that the assumption of axial symmetry may, in general, not hold. Similar conclusions have been reached by other investigators in studies of paramagnetic metal binding to model systems. We have included the three Co^{2+} coordinates in an eight-parameter fit of the Co^{2+} shift data. The Co^{2+} position obtained from this fit is in statistical agreement with the position inferred from x-ray data. Thus, the analysis of shift data may furnish a means for determining the site of metal complexation in macromolecules whose structure has been determined by x-ray crystallography.

The well-documented success of proton nuclear magnetic resonance (^{1}H NMR)[1] spectroscopy to provide configurational and conformational information about organic molecules led to the applications of this method to studies of the structures of proteins in solution (Dwek, 1973; James, 1975; Wüthrich, 1976). Such studies continue to be hampered by the fact that even with the development of high-field superconducting spectrometers, ^{1}H NMR spectra of proteins are complex and only partially resolvable because of the large number of protons present in protein molecules. In 1969, a method was introduced which could potentially deal with this inherent problem. McDonald & Phillips (1969) showed that the addition of Co^{2+} to a solution of HEW lysozyme produced differential shifts in the upfield portion of the protein spectrum, leading to the resolution of previously overlapping peaks. Hinckley (1969) found that the dipyridine adduct of europium(III) tris(dipivalomethane) produced stereospecific shifts in the protein spectrum of cholesterol. These two sets of observations stimulated a large number of reports dealing with "shift reagent" research (for reviews see: Cockerill et al., 1973; Reuben, 1973; Sievers, 1973), in which the specific electron–nuclear interactions produced by paramagnetic ions were used to alter the ^{1}H NMR spectra of organic molecules.

In paramagnetic complexes, these electron–nuclear interactions are manifested in two changes in the NMR spectrum of the ligand molecule: chemical-shift perturbations and enhanced relaxation rates (for reviews see: Eaton & Phillips, 1965; De Boer & van Willigan, 1967; Webb, 1970; La Mar et al., 1973). The total shift observed in the resonance of a given nucleus in the presence of paramagnetic ions can be expressed as:

$$\Delta = \Delta_{CF} + \Delta_C + \Delta_D \quad (1)$$

where Δ_{CF} is the complex formation shift and is diamagnetic in origin, Δ_C is the shift arising from the Fermi contact interaction (a through-bond effect), and Δ_D is the shift arising from the dipolar or pseudocontact interaction (a through-space effect). The value of Δ_{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 electron–nuclear interactions are effectively averaged out. For complexes in which the susceptibility is anisotropic, the dipolar shift is given by (McConnell and Robertson, 1958):

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

where K_1 and K_2 are constants related to elements of the magnetic susceptibility tensor of the metal ion, and r, θ, ϕ are the spherical polar coordinates of the nucleus relative to the principal magnetic axis system of 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.

It is clearly seen from eq 2 that dipolar shifts are stereospecific in origin, containing both a distance and angular dependence. The utilization of dipolar shifts has ranged from the removal of accidental shift degeneracy to quantitative computer-based schemes which fit these shifts to a given structure for the paramagnetic complex using the axial form of eq 2 (see Willcott & Davis, 1975, and references cited therein).

Many biologically interesting molecules have diamagnetic divalent cations (Mg^{2+}, Ca^{2+}, Zn^{2+}, etc.) as cofactors or inhibitors. Since these metals, in general, produce small perturbations in the ^{1}H NMR spectrum of the biological molecules, paramagnetic analogues of these metals which produce larger perturbations are often employed (see Chapter IV of Dwek, 1973; Chapter VI of Wüthrich, 1976, and references cited therein). Thus, the trivalent lanthanides have been used as replacements for Ca^{2+}, Co^{2+} as a replacement for Zn^{2+}, and Mn^{2+} for Mg^{2+}. In those proteins which do not normally bind metal ions, it has been demonstrated that chemical modifica-

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[1] Abbreviations used: HEW, hen egg white; NMR, nuclear magnetic resonance.

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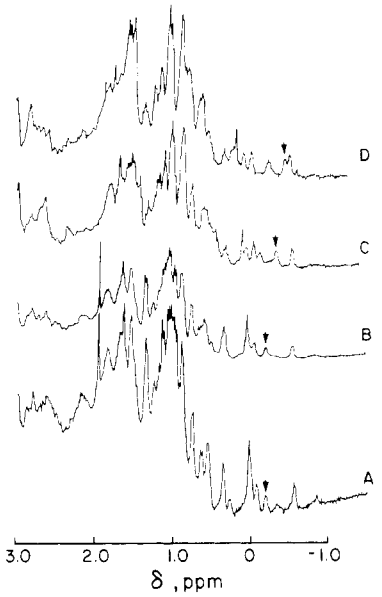

FIGURE 1: The upfield region of the spectrum of lysozyme (3.35 mM) at pD_c of 5.5 and 55 °C with (A) 25 mM Zn^{2+} present, (B) no metals present, (C) 12.5 mM Co^{2+} present, and (D) 32.5 mM Co^{2+} present. The arrow indicates the Ile-98 CH_3 δ_1 resonance.

tions can be made in order to introduce a specific metal binding site (Marinetti, 1975; Marinetti et al., 1975, 1976, 1977).

The use of lanthanide ions as probes in biological systems was introduced by the Oxford enzyme group in their study of mononucleotide conformations in solution (Barry et al., 1971). This work was later extended to the cyclic nucleotide phosphates (Barry et al., 1974). In these two reports, the paramagnetic shifts and relaxation rates induced by lanthanide ions were compared with calculated values obtained by means of a computer program which sampled all possible conformations by allowing free rotation around the appropriate bonds and excluding those conformations which violated constraints imposed by van der Waals contacts. In this way, a relatively small number of conformations were obtained for which the observed and calculated results agreed within acceptable limits. This group also began an extensive study of the interactions of lanthanide ions with HEW lysozyme (Campbell et al., 1973a,b, 1975a,b) in which the shifts and relaxation rates induced by these ions were analyzed in conjunction with the known x-ray structure of the protein.

Their analysis was based on the assumption that the axial form of eq 2 is the correct model for the interpretation of dipolar shifts, a conclusion drawn from the observation that shift ratios between two different proton resonances in lysozyme remained constant over a series of lanthanide ions. However, as has been pointed out by Marinetti et al. (1975), this observation does not guarantee axial symmetry. By fitting their data to both the axial and nonaxial forms of eq 2, we have tested the validity of the assumption made by the Oxford group. Using a statistical hypothesis test (vide infra) we concluded with 97.5% confidence that the axially symmetric model for Nd^{3+} and Ce^{3+} does not hold (Agresti et al., 1977).

The present study extends this analysis to transition metal complexes and presents a general methodology with which to analyze dipolar shifts induced by paramagnetic ions. In particular, we apply this method to the shift perturbations induced by Co^{2+} in HEW lysozyme. This molecule offers several clear advantages as a system with which to illustrate our approach. Lysozyme is a low molecular weight enzyme (~14 700) with a known amino acid sequence (Jolles et al., 1964; Canfield & Liu, 1965). The mechanism of its action has been established (Rupley, 1967; Smith et al., 1973); see, however, Warshel & Levitt (1976). The x-ray crystallographically determined structure for native lysozyme (Blake et al., 1967; Phillips, 1967; Imoto et al., 1972) as well as Fourier difference maps for various metal-lysozyme complexes have been reported (Kurachi et al., 1975; Yonath et al., 1974). In solution, lysozyme has been shown to bind polyvalent cations (McDonald & Phillips, 1969; Gallo et al., 1971; Campbell et al., 1973a,b, 1975a,b). The metal is complexed to the side-chain carboxyl groups of Glu-35 and Asp-52 (Ikeda & Hamaguchi, 1973). Studies of the effects of transition metal binding on the photooxidation of lysozyme tryptophan residues indicate that the binding of neither Co^{2+} nor Zn^{2+} significantly perturbs the overall tertiary structure of the enzyme (Jori et al., 1971). For the purpose of making diamagnetic corrections in this study, the interactions of Zn^{2+} with lysozyme were also monitored.

Materials and Methods

Crystalline salt-free HEW lysozyme (three times recrystallized) was purchased from Worthington Biochemical Corporation (Freehold, N.J.). The protein was dissolved in D_2O and its concentration determined spectrophotometrically (Sophianopoulos et al., 1962). Stock solutions of $CoCl_2$ and $ZnCl_2$ were prepared by dissolving the appropriate weight of these salts in the lysozyme solution. 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 Bruker HX-270 spectrometer operating in the FT mode. Each spectrum was the sum of 128 scans. All spectra were recorded at a pD_c of 5.5 (pH meter reading + 0.5) 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.

Results and Discussion

Binding Parameters from Co^{2+} Shift Data. We have assumed that Co^{2+} forms 1:1 complexes with lysozyme.[2] A similar assumption was made by McDonald & Phillips (1969). For such a complex the binding process can be written as:

$$M + L \rightleftharpoons ML \tag{3}$$

with an association constant, K, given by

$$K = [ML]/[M][L] \tag{4}$$

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 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. This situation is clearly illustrated by Figure 1 where the upfield region of lysozyme is shown in the presence of Zn^{2+} and Co^{2+}. From this figure it is clear that the addition of Co^{2+} to a solution of lysozyme results in the observation of one set of shifted lysozyme resonances. The magnitudes of the shift perturbations

[2] We have found that Co^{2+} inhibits lysozyme activity (Ostroy et al., 1978). The inhibition data were consistent with a binding model in which there is a single metal binding site causing complete inhibition.

TABLE I: Experimental Co^{2+}-Induced Shifts and Calculated Values for Various Methyl Groups of Lysozyme.

Resonance	Limiting shifts (Hz)[a]	Calcd for axial symmetry[b]	Calcd for non-axial symmetry[b]	Coordinates[c] r (Å)	θ (deg)	ϕ (deg)
Leu-8 CH_3 δ_1	37.5	37.5	54.7	13.9	137	277
Leu-8 CH_3 δ_2	37.5	23.1	32.5	15.7	133	265
Leu-17 CH_3 δ_1	13.3	18.2	14.7	16.9	137	216
Leu-17 CH_3 δ_2	35.5	22.7	30.0	15.8	139	231
Ile-88 CH_3 γ_2	8.6	7.4	7.7	15.9	117	257
Ile-88 CH_3 δ_1	17.7	12.9	16.4	17.1	128	250
Ile-98 CH_3 γ_2	−136.7	40.4	−119.8	11.8	112	183
Ile-98 CH_3 δ_1	−132.3	−4.3	−122.2	11.7	106	203
Met-105 CH_3 ϵ	104.8	65.0	120.0	11.5	152	190
Val-109 CH_3 γ_1	−140.2	−167.1	−106.9	6.2	99	98
Val-109 CH_3 γ_2	−429.9	−333.6	−465.6	3.3	109	110

[a] The 15% error observed for Ile-98 CH_3 δ_1 (cf. text and Figure 3) was used for all shifts, except for Leu-8 CH_3 δ_1 and δ_2, whose shifts were not resolved, where $\sqrt{2} \times 15\%$ was used. [b] Using parameters from best fit (cf. Table II). [c] In the magnetic axis system, defined by parameters from the nonaxial fit. In this system active-site coordinates are: (r, θ, ϕ) = Asp-52 O δ_2 (4.42, 47, 231); Glu-57 O (5.71, 91, 218); Glu-35 O (4.85, 114, 301); and Glu-35 O ϵ_1 (2.39, 153, 271).

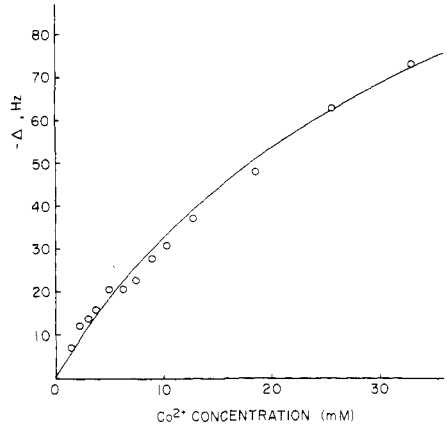

FIGURE 2: The variation in the chemical shift of the Ile-98 CH_3 δ_1 resonance (referenced to its unperturbed position) with Co^{2+} concentration. Points represent experimental data and the line was calculated using the best fit parameters (vide infra).

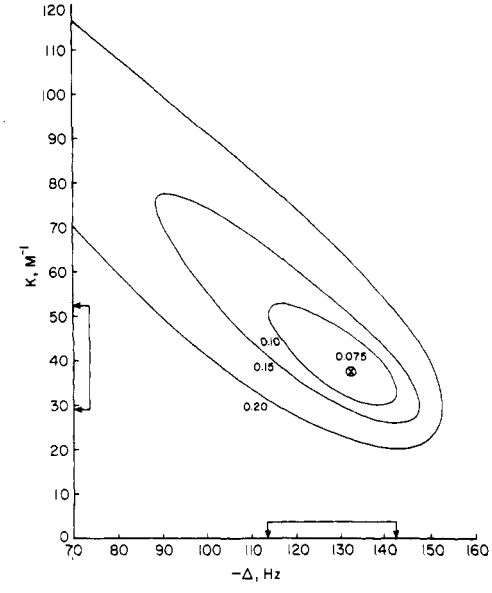

FIGURE 3: The effect of variations in K and Δ on S. The contours represent those combinations of K and Δ which yield the S value indicated. Confidence intervals for K and Δ are indicated with the arrows in the axes (vide infra).

are clearly dependent on the Co^{2+} concentration. Such chemical-shift perturbations are given by:

$$\delta^i_{obsd} = [ML]\Delta_i/L_T \quad (5)$$

where δ^i_{obsd} is the observed chemical shift of the ith nucleus, referenced to the chemical shift in that same nucleus with no metal present (δ^i_0), Δ_i is its limiting chemical-shift value in the complexed state also referenced to δ^i_0, and L_T refers to the total lysozyme concentration present. Since the presence of Zn^{2+} causes negligible perturbations in the lysozyme spectrum (see Figure 1A), the Δ_{CF} term in eq 1 can be neglected.

The variation in the observed chemical shift of the δ_1 methyl resonance of isoleucine-98 (Ile-98 CH_3 δ_1) with increasing Co^{2+} concentration is shown in Figure 2. Using a simplex algorithm (O'Neill, 1971), these data were fit to two parameters, K and Δ (eq 4 and 5), by minimizing the quantity S given by:

$$S = \left[\sum_{i=1}^{N} (\delta^i_{obsd} - \delta^i_{calcd})^2 \Big/ \sum_{i=1}^{N} \delta^i_{obsd}{}^2 \right]^{1/2} \quad (6)$$

where N is the number of data points and δ^i_{calcd} refers to the calculated shifts. Estimates of the error limits for these two parameters were obtained by varying K and Δ around their best-fit values and establishing an interval for each parameter for which the S value was within a factor of 2 of the minimum S value (0.075). This procedure is illustrated in Figure 3. Using these methods, values of $K = 37 \pm 15$ M^{-1} and $\Delta = 132 \pm 18$ Hz were obtained from the data shown in Figure 2.

For the other assignable resonances overlap with other peaks made chemical-shift measurements difficult over parts of the range of cobalt concentrations. Limiting values for their chemical shifts were computed from the value of K obtained for Ile-98 CH_3 δ_1, eq 5, and the values of their observed shifts over the part of the Co^{2+} concentration where overlap did not occur.[3] The error limits for the limiting shifts of these resonances were similar to those obtained for Ile-98 CH_3 δ_1 (±15%). In general, our shift results given in Table I were qualitatively similar to those reported by McDonald & Phillips (1969).

Analysis of Shift Data. It should be noted that if the coordinates for both the metal ion and the nuclei being observed

[3] This was accomplished by a linear least-squares fit of the observed shift to the values of $[ML]/L_T$ calculated from K. The slope of such a fit is Δ.

TABLE II: Statistics and Parameters[a] for Co^{2+}–Lysozyme Shift Data.

Model	χ^2	R factor	Ratio	10^4K_1 Hz (Å^3)	K_2/K_1	ϕ_e (deg)	θ_e (deg)	ψ_e (deg)
Axial[b]	41.0	0.573	3.47	10.3 ± 0.5		3.9 ± 0.9	62.0 ± 0.3	
Nonaxial[c]	4.53	0.165		−15.1 ± 0.9	0.81 ± 0.03	72.0 ± 2.4	67.5 ± 0.8	−73.2 ± 3.2
Nonaxial[d]	2.93	0.094		−10.4 ± 1.4	0.50 ± 0.18	74.4 ± 1.9	53.9 ± 5.1	−86.3 ± 10.9

[a] The error estimates are those generated by the Taylor's approximation in the least-squares algorithm. [b] A second minimum (χ^2 = 54.1; R factor = 0.658) was found for $K_1 = (-3.1 \pm 0.2) \times 10^4$ Hz Å^3, $\phi_e = 110 \pm 2°$, $\theta_e = 67 \pm 5°$. [c] Co^{2+} position fixed (see footnote 4) at (7.869, 24.804, 18.601). [d] Co^{2+} position variable, determined by the fit to be (6.9 ± 0.4, 23.7 ± 0.6, 20.6 ± 0.8).

are known, the calculation of shifts using the axial form of eq 2 requires the specification of three parameters: K_1 and two Eulerian angles, ϕ_e and θ_e (Goldstein, 1959). If the nonaxial form of eq 2 is used, the specification of two additional parameters, K_2 and ψ_e, is required. In the present analysis of the Co^{2+}-induced shifts, we make use of the x-ray crystallographically determined coordinates of native lysozyme (Blake et al., 1967; Phillips, 1967) together with the coordinates for the Co^{2+} inferred[4] from the data reported by Kurachi et al. (1975). The use of atomic coordinates of the native lysozyme is justified by x-ray data which indicate that the conformational perturbations associated with metal binding to this enzyme are confined to the immediate vicinity of the metal binding site. In Table I there are no residues in such close proximity to the metal binding site which also justifies the neglect of the Δ_C term in eq 1. Methyl hydrogen coordinates were calculated assuming a C–H bond distance of 1.09 Å using a tetrahedral geometry. The methyl group was represented by the average position of the three hydrogen atoms.

All shift computations employed a versatile least-squares fitting algorithm based on the Marquardt search technique (Marquardt, 1963) written in Speakeasy (Cohen, 1976). This algorithm combines a steepest descent routine with a first-order Taylor's approximation which provides convergence even for arbitrary initial parameter estimates (steepest descent feature) as well as error estimates for the parameter values obtained at the best-fit convergence (Taylor's approximation). Convergence was considered to be complete when parameter values were stable to 1 part in 10^5.

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

$$\chi^2 = [1/(N_d - N_p)] \sum_{i=1}^{N_d} [(f_i - y_i)/\sigma_i]^2 \qquad (7)$$

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 at each point, and σ_i is the assumed error. The second parameter, called the "agreement factor", R, is given by Hamilton (1965), Willcott et al. (1972), and Davis & Willcott (1972) as:

$$R = \left[\sum_i [(f_i - y_i)^2/\sigma_i] \Big/ \sum_i (y_i^2/\sigma_i) \right]^{1/2} \qquad (8)$$

[4] Kurachi et al. (1975) give distances from Mn^{2+} to Asp-52 O δ_2, Gln-57 O, Glu-35 O, and Glu-35 O ϵ as 4.57, 5.57, 4.81, and 2.52 Å, respectively. Together with the x-ray coordinates of these active-site atoms (8.955, 21.851, 21.706), (5.162, 20.803, 21.648), (6.398, 22.127, 14.829), and (5.801, 24.411, 17.479), respectively, least-squares optimization with respect to calculated distances places the Co^{2+} ion at (7.9 ± 0.1, 24.8 ± 0.1, 18.6 ± 0.1), within 0.15 Å of each of the above distances.

The use of significance tests based on the R factor has been described in detail (Hamilton, 1965) in the assessment of the reliability of hypotheses regarding the results of crystallographic studies. This approach has been especially useful in evaluating various structural models which differ in the number of model-dependent parameters present in each (e.g., anisotropic vibration vs. isotropic vibration). The utility of this approach in the analysis of lanthanide shift reagent data has been demonstrated (Willcott & Davis, 1975). Briefly, the procedure is as follows. The data are fit to each of several models and a minimum R value is obtained for each fit. For each pair of models, an R-factor ratio is computed (>1, by convention). The following null hypothesis is formulated: the two models fit the data with equal precision. A confidence interval at which this hypothesis can be rejected is established by reference to Hamilton's tables (1965). Rejection of a hypothesis at a given confidence interval α% means that one risks rejection of the true hypothesis α% of the time. It must be pointed out that this method is *statistical* in nature and as such can only ascertain which model tested fits the data *most precisely*.

The Co^{2+} shift data in Table I were fit to both forms of eq 2 using the Marquardt algorithm. The shifts calculated from the best fit axial and nonaxial parameters are given in Table I. The "best-fit" parameters are summarized in Table II. For the nonaxial fits, there are numerous sets of the five parameters which generate the same calculated values of the shifts. These include: three sets from an arbitrary labeling of the z axis; for each of these, two sets from the direction of the z axis; four sets arising from 90° 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 set of parameter values summarized in Table II: the z axis is chosen to maximize $|K_1|$, which forces $\rho < 1$; the x and y axes are chosen so that $\rho > 0$; and the two Euler angles θ_e and ψ_e are restricted by requiring $0 < \theta_e < \pi/2$ and $-\pi/2 < \psi_e < \pi/2$.

From Table I, it is clear that there are several discrepancies between the observed shifts and those calculated from the axial model. The most obvious of these is the disagreement in the results for the Ile-98 methyl groups. These inconsistencies are reflected in the rather large agreement factor of 0.573 (cf. Table II) obtained for the axial fit. For the nonaxial parameters, the differences between the observed and calculated shifts obtained fall in the range of the experimental errors of the observed values (~15%). The smaller agreement factor of 0.165 obtained for this fit reflects the greater consistency of the nonaxial model with the data.

In order to statistically evaluate the confidence interval with which the axial model can be rejected, we performed a hypothesis test on the basis of the R-factor ratio for the two models. Reference to Hamilton's tables (1965) for this hypothesis of dimensionality 5 (5-parameter fit vs. 3-parameter

fit)[5] with 6 degrees of freedom (11 observations minus 5 parameters) indicates that the R-factor ratio required for rejection of this hypothesis with 99% confidence is 3.459. Since the R-factor ratio obtained for our hypothesis is 3.473 we conclude with greater than 99% confidence that the most general form of eq 2 must be used in analyzing the Co^{2+}-lysozyme shift data. Using the best-fit nonaxial parameters given in Table II in conjunction with the appropriate x-ray coordinates, we can now calculate shifts for any residues in lysozyme. Thus, it may be possible to assign other resonances in lysozyme provided that these resonances can be identified with a particular type of amino acid.

We have examined the effect of including the three Co^{2+} coordinates as variable parameters in the nonaxial fit. Because of the larger number of parameters (eight) now present in this fit, there is no guarantee of convergence. The Marquardt algorithm was initialized using the best-fit parameter values for the nonaxial fit (Table II) along with the Co^{2+} position[4] used previously. The fit converged with a χ^2 of 2.93 and an R factor of 0.094. The Co^{2+} position obtained from this fit (6.9 ± 0.4, 23.7 ± 0.6, 20.6 ± 0.8) Å is in statistical agreement with the position inferred from the x-ray data of Kurachi et al. (1975). The parameter values describing the magnetic susceptibility tensor of the Co^{2+} ion in this fit are given in Table II. There is qualitative agreement between these parameter values and those obtained using a fixed Co^{2+} position. There are generally larger errors and a higher degree of correlation observed in the former set of parameters, reflecting the smaller number of degrees of freedom in this fit. This set of parameters corresponds to a local minimum since, when the Marquardt algorithm was initialized using arbitrary Co^{2+} positions, the fit converged to other sets of parameters which seemed to have little physical significance. We suggest that this behavior arises from the small number of degrees of freedom in the analysis, i.e., the small number of limiting shifts in Table I. This number might be expanded by extending this study to the downfield region of the lysozyme spectrum or by including Co^{2+}-induced perturbations of the ^{13}C spectrum of lysozyme.

In summary, we have presented a general methodology with which to analyze dipolar shifts induced by paramagnetic ions. In the particular example which we have used, a hypothesis testing scheme was employed to statistically evaluate the relative precision of two models, the axial and nonaxial forms of eq 2. The axial model was rejected with greater than 99% confidence. In a previous analysis of the lanthanide-induced shifts of lysozyme (Agresti et al., 1977), we reached the conclusion that the axial model could be rejected with greater than 97.5% confidence. On the basis of qualitative evidence, similar conclusions have been reached in the studies of lanthanide binding to *N*-acetyl-L-3-nitrotyrosine ethyl ester (Marinetti et al., 1975) and lanthanide binding to salicylaldehyde and nitrophenol (Reuben, 1976). We therefore suggest that the assumption of axial symmetry must be scrutinized carefully in any applications of paramagnetic ions as "shift reagents" in biological systems.

We wish to point out that the hypothesis testing scheme presented here can also be employed in the manner described by Willcott & Davis (1975) and Davis et al. (1973) in their analyses of lanthanide-induced shifts in organic molecules. That is, several possible structures can be tested against one set of shift data in order to ascertain which structure fits the data most precisely. In order for this procedure to yield meaningful results, the structures tested must be selected on the basis of information obtained using independent methods (e.g., x-ray data or potential energy calculations). Once a structure has been established using the correct form of eq 2, the dipolar shifts for the molecule can be calculated. These calculated shifts may, in many cases, aid in making assignments of resonances in the NMR spectrum of the molecule. The analysis of shift data described in this report as well as the fitting of relaxation data outlined earlier (Agresti et al., 1977) may furnish a means for determining the site of metal complexation in macromolecules whose native structure has been determined by x-ray crystallography, provided that there is evidence that such complexation does not markedly alter the overall tertiary structure of the macromolecule.

[5] When comparing R-factor ratios obtained from two models containing a different number of model-dependent parameters, Hamilton chose to set the dimensionality and the number of degrees of freedom in the most conservative way. Thus, we make the dimensionality five instead of three.

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Effects of Hofmeister Salts on the Self-Association of Glucagon†

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ABSTRACT: The trimerization constants of glucagon at pH 10.6 in 0.76 M K_2HPO_4 have been calculated from circular dichroism data between 5 and 50 °C. The free energy, enthalpy, and entropy of transfer have been evaluated from the current results and published data in 0.20 M phosphate. The free energies of transfer from 0.20 to 0.76 M phosphate are negative at all temperatures investigated. The negative free energies of transfer are derived completely from an increase in the entropy of transfer, since the enthalpy of transfer is less favorable at all temperatures. These parameters are compared with those of various model groups and compounds: CH_2, peptide, methane, ethane, and the 1–13 N-terminal fragments of ribonuclease. The effects of fluoride and chloride on the self-association of glucagon have been compared with that of phosphate at 25 °C. These effects are consistent with the binding of approximately one molecule of salt to the trimer and a systematic decrease in the number of water molecules bound to the trimer compared to the monomer for the series K_2HPO_4, KF, and KCl.

In dilute solutions glucagon is largely unstructured, with few intramolecular contacts (Blanchard and King, 1966). At moderate concentrations a largely α-helical trimer is formed in alkali (Gratzer and Beaven, 1969). Cross-linking experiments with dimethyl suberimidate have demonstrated that the associated species is a trimer; no dimers or hexamers were found (Gratzer et al., 1972). It is also known that glucagon is a trimer in its crystalline state (Sasaki et al., 1975). Previous measurements of the self-association behavior of glucagon are consistent with a monomer $\rightleftharpoons$ trimer equilibrium (Formisano et al., 1977).

Since the self-association in alkali can be conveniently measured by several methods and the structure of the trimer is known from x-ray analysis (Sasaki et al., 1975), this association can serve as a useful model to evaluate the influence of the solvent composition on the equilibria involved in protein interactions. Since the x-ray data reveal that the trimer is

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