the effect of nonbonded interactions on the ^{13}C shift along each bond of each position is pictorially represented by placing "+" where the corresponding coefficient from the multiple regression is positive and a "-" where it is negative. Unfortunately, it will have to await further study before any predictive rules can be developed as to which direction these induced dipoles will take in more general cases. We have stated earlier (vide infra) that the para-carbon shift remains unchanged throughout this series of compounds. If these ^{13}C shifts are due to charge polarization brought on by van der Waals interactions, then the lack of change in the para-carbon shift indicates that the π system is unaffected and that the polarization occurs solely in the σ system. For instance, the calculated net ^{13}C shift for C_4 of pentaphenylethane due to nonbonding forces along its bonds is -7.75 ppm, and for 1,2-diphenylethane this value is -13.29 ppm. Thus in going from 1,2-diphenylethane to pentaphenylethane the ortho-carbon (C_4) is shifted downfield by 5.54 ppm due to the change in nonbonding interactions in these two molecules. If this downfield shift is due to an accumulation of positive charge on the ortho carbons, then simple resonance theory[25] tells us that a similar amount of positive charge should buildup on the para-carbon and thus we would expect a downfield shift of its ^{13}C signal. Theory[25] further tells us that, to a first approximation, the σ and π systems of an aromatic ring are independent of one another. Thus it would appear the polarization of electrons is perturbing only the σ-electron distribution and not significantly effecting the π electrons. One exception to the observed sign alternation is the α-α' bond. Here a stretching force causes an upfield shift of both tetrahedral carbons. This is a consequence of the symmetry of these compounds; thus if 1,1,2,2-tetraphenylethane were stretched along the α-α' bond, a dipole is not expected to be produced since the two carbons are by symmetry the same. In a previous paper[26] we had endeavored to correlate the ^{13}C shift of α-substituted benzyl cations with calculated (CNDO/2) charge densities. While the ^{13}C shifts of the para position lay close to the least-squares line ($r = 0.980$), the shifts for the other positions deviated considerably. One possible reason now offers itself: the electron densities of the α, ipso, ortho, and meta positions conceivably are altered by van der Waals interactions within the molecule, an effect which is not dealt with explicitly at the CNDO/2 level.

In summary we have shown (1) the induced polarization of electrons along each bond by steric interactions previously postulated by Grant and Cheney, (2) that van der Waals interactions are equally important for all positions, and (3) a general treatment for elucidating steric effects in organic compounds using ^{13}C NMR. Our primary purpose here has not been to reproduce experimentally found ^{13}C shifts but to ferret out one of the factors that determines them. It is hoped that ultimately a model will be devised in which a term as vague as a "substituent effect" can be eliminated and replaced with one reflecting the change in the electronic properties induced in a molecule by the substituents and thus make it possible to have a model based on experimental observations as interpreted by theory to predict the electronic as well as steric properties of a molecule.

Experimental Section

^{13}C Spectroscopy. The study was carried out by using a Varian Associates Model XL-200 spectrometer; the instrument and techniques used are analogous to those described previously for the XL-100.[27] All reported shifts are at ambient temperature in $CDCl_3$ (0.5 M) and are referenced to external capillary tetramethylsilane.

Preparation of Phenylethanes. 1,1,1,2,2-Pentaphenylethane was prepared according to the method of Bachmann.[28] The purification was modified by washing the crude product with diethyl ether before recrystallization. The yield was 85.7%; mp 180 °C (lit. 182-185 °C). Anal. Calcd: C, 93.65; H, 6.34. Found: C, 93.77; H, 6.21. 1,1,1,2-Tetraphenylethane was prepared by the procedure of Bachmann and Cockerill,[29] with a yield of 83.4%; mp 142 °C (lit.[30] 143.6 °C). 1,1,1-Triphenylethane was also synthesized by the method of Bachmann,[29] the yield was 93.2%; mp 94 °C (lit.[28] 94.9 °C). All other compounds were commercially available and used without further purification.

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Proton Nuclear Overhauser Effects and Protein Dynamics

E. T. Olejniczak, F. M. Poulsen, and C. M. Dobson*

Contribution from the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received January 26, 1981*

Abstract: The rates of direct nuclear cross-relaxation between pairs of protons of three amino acid residues of hen lysozyme (Trp-28, Ile-98, and Met-105) have been obtained at 270 and 498 MHz by analyzing the time dependence of nuclear Overhauser effects. The proton pairs were chosen to have short internuclear distances fixed by the geometry of the residues themselves. The measured cross-relaxation rates were compared with rates calculated on the assumption that the protein molecule behaves as a rigid body tumbling isotropically in solution with a rotational correlation time defined from independent studies. Differences between the measured and calculated rates were attributed to the effects of significant internal motions. It was demonstrated that the proton cross-relaxation data can define the extent of specific types of internal motion. As an example, limits were placed on the magnitude of subnanosecond fluctuations of individual side-chain torsional angles by using a restricted diffusion model. The consequences of the experiments for investigation of molecular dynamics, for structural studies, and for other relaxation phenomena are discussed.

Saturation of a specific resonance in the nuclear magnetic resonance spectrum of a molecule can give rise to changes in the intensities of other resonances through the nuclear Overhauser effect.[1] In a system of dipolar coupled spins, the magnitude of the effect on a given spin following saturation of another for a fixed length of time depends on the frequencies of the motions

* To whom correspondence should be addressed at the Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR, England.

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of the spins and on the inverse sixth power of the distances separating pairs of spins. For a large molecule such as a protein, nuclear Overhauser effects contain much information about structural and dynamical properties.[2-7] An analysis of the effects may be very complex, however, as it requires in general consideration of many internuclear distances and of a model representing the rates of molecular motion by correlation times.[8-10] Difficulties can be minimized if the nucleus on which an effect is observed is very close to the one which is saturated and is isolated from other spins. Such a situation may be realized, for example, in ^{13}C or ^{15}N NMR when ^{1}H spins directly attached to these nuclei are saturated.[11,12] Because the distances between the observed and saturated spins are known with high accuracy, correlation times describing the motion of the C–H or N–H vectors can be extracted by using a model which considers two spins only. In conjunction with other relaxation measurements these studies have provided valuable information about dynamics of proteins.[11-15] They do not, however, provide information about distances between residues or about the relative motion of one residue with respect to another, and the application of these methods is also restricted by the low sensitivities of the nuclei.

Proton NMR has the advantage of high sensitivity and generally easier assignment procedures.[16] Where both saturated and observed spins are protons, however, they cannot be considered as isolated and saturation may spread efficiently through the system, giving rise to the phenomenon of spin diffusion.[8,10,17] One way of overcoming this problem is to study Overhauser effects at very short times after saturation.[6,7,9,10] Under these limiting conditions the magnetizations of spins other than that saturated are not significantly different from their equilibrium values, and the Overhauser effects, at least on protons closest to the saturated spin, can be interpreted by using a two-spin approximation. This selective double-resonance method therefore has a real advantage in proton NMR over measurements of relaxation rates by non-selective pulse techniques.

Recently, we have shown[7,18] that relative nuclear Overhauser effects measured shortly after saturation of assigned resonances in the proton NMR spectrum of the protein lysozyme in solution can be directly correlated with inverse sixth powers of interproton distances from the crystal structure to an accuracy of about ±1 Å. This technique has, therefore, considerable utility in structural studies of proteins in solution. In the present work, the dependence of Overhauser effects on the length of time of saturation of resonances of three residues of lysozyme has been studied in detail. The residues were chosen such that each saturated proton has a close neighbor at a known distance fixed by the geometry of the residue. The motivations for this study were threefold. First, the experiments permit the feasibility and accuracy of a two-spin analysis to be investigated. Second, from the time dependence of the Overhauser effects, rates of cross-relaxation between pairs of protons can be obtained. Comparison of these rates with those calculated on the basis of independent measurements of the correlation time for overall rotation of the protein molecule suggests a method for investigation of the internal motion of individual residues of a protein. The method is illustrated here by calculating with a simple model the effects on cross-relaxation rates of fast internal motions of the type predicted to occur in recent theoretical studies of protein dynamics.[19-21] Third, the consequences of the effects of motion on cross-relaxation rates for distance measurements between protons of different residues can be examined.[7]

Methods

Proton NMR spectra were recorded at 270 MHz by using a Bruker spectrometer and at 498 MHz by using the home-built spectrometer at the Francis Bitter National Magnet Laboratory.[22] Nuclear Overhauser effects were measured from peak areas by using interleaved difference spectroscopy[4,7,9] with presaturation pulses of defined lengths between 0.02 and 0.20 s. Delays of at least 3.0 s were included between scans to permit relaxation of the system. Typically, a total of 1000 scans resulted in an adequate signal-to-noise ratio. The experimental conditions were adjusted to approximate closely to instantaneous saturation, by using high irradiation powers.[23] The power level in each experiment was chosen so that the intensities of the observed effects were at a maximum value in the difference spectrum. Considerable care was taken to establish that higher saturation powers did not affect the time development and also that non-selective effects were insignificant.[23,24]

Lysozyme from hen egg-white (E.C.3.2.1.17) was obtained from the Sigma Chemical Co. (grade I) and prepared as described previously.[7] Samples for NMR were 7 mM in lysozyme in D_2O at pH 3.8, and spectra were run at 32 °C. Removal of oxygen by several freeze–thaw cycles and of any stray paramagnetic ions by addition of 0.1 mM EDTA had no observable effects on measured Overhauser effects. Chemical shifts are given in parts per million (ppm) downfield from the methyl group resonance of 4,4-dimethyl-4-silapentanesulfonate and were measured relative to internal standards of acetone and dioxan. Assignments in the lysozyme spectrum have recently been summarized.[7]

Computation was carried out by using a Vax 11/780 computer of the Harvard University chemistry department. Proton coordinates were generated in a standard manner from the refined X-ray crystal structure of tetragonal lysozyme[25] by using a program adapted from one kindly supplied by H. J. C. Berendsen. The multispin calculations were carried out by using a program based on that described by Bothner-By and Noggle.[8] Energy calculations were carried out by using programs developed by Karplus and co-workers.[26]

Results

Cross-Relaxation Rates. In Figure 1 are shown difference spectra obtained at 498 MHz resulting from saturation, with a

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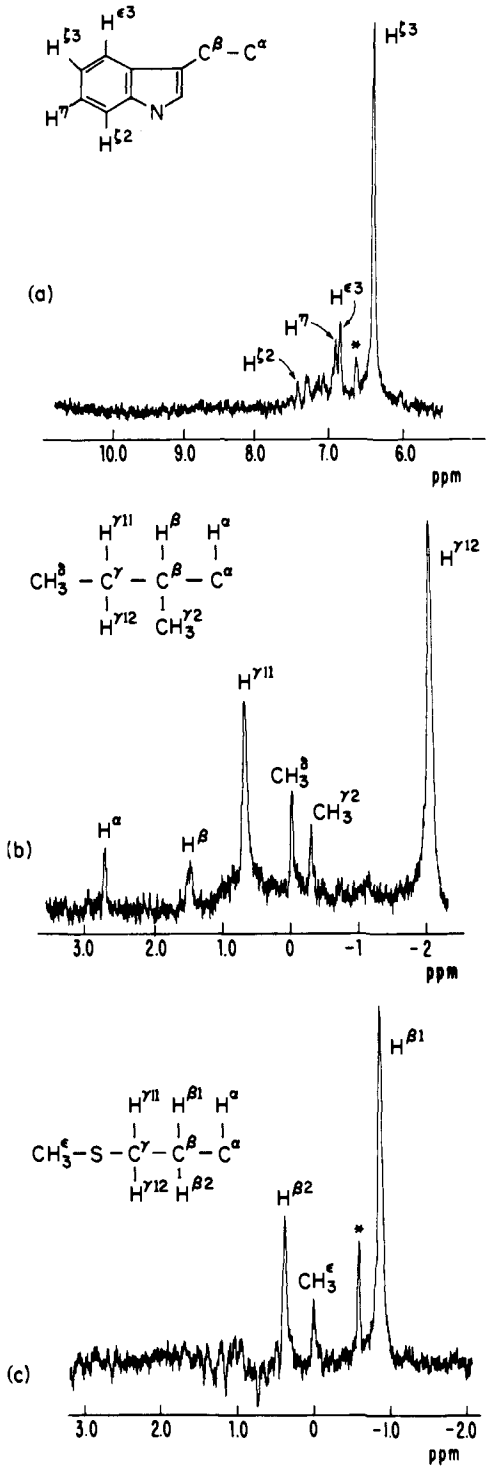

Figure 1. Difference spectra at 498 MHz resulting from the application of a pulse of length 80 ms at the resonant frequencies of (a) $H^{\zeta 3}$ of Trp-28, (b) $H^{\gamma 12}$ of Ile-98, and (c) $H^{\beta 1}$ of Met-105. The irradiated resonance is at the right-hand side of each spectrum, and other assignments are as indicated.[7] Peaks labeled with an asterisk arise from nonselective effects. These effects make no contribution to the intensities of other peaks in the difference spectra.

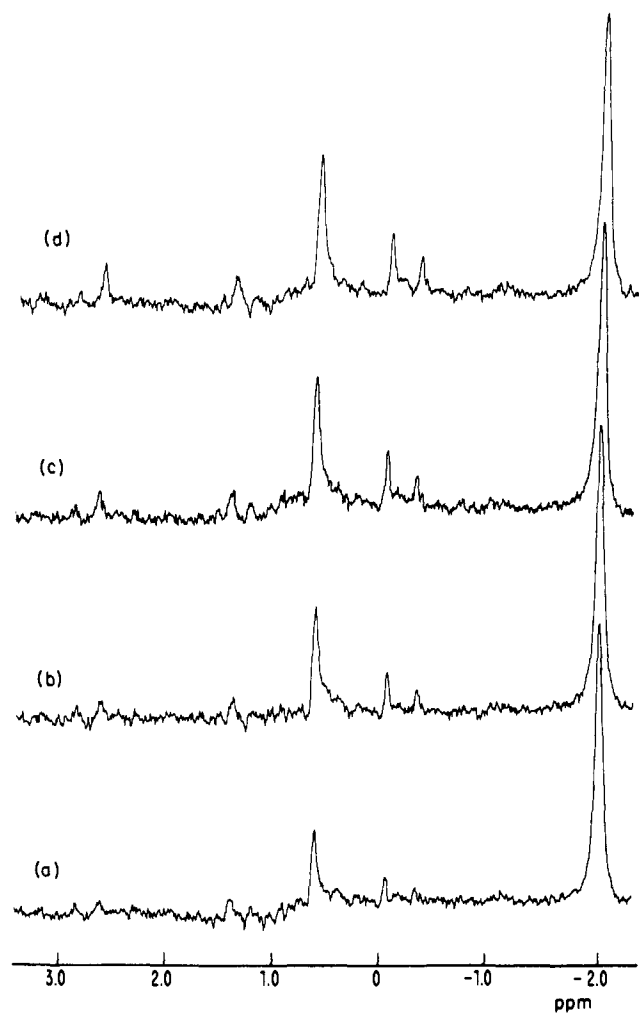

Figure 2. Difference spectra at 498 MHz, see Figure 1, resulting from irradiation at the resonant frequency of $H^{\gamma 12}$ of Ile-98 for (a) 20 ms, (b) 40 ms, (c) 60 ms, and (d) 80 ms.

pulse of 80 ms in length, of resonances from each of the three residues studied, Trp-28, Ile-98, and Met-105. Saturation of the $H^{\zeta 3}$ resonance of Trp-28 (6.28 ppm) results in large Overhauser effects on the resonances of the two adjacent ring protons $H^{\epsilon 3}$ (6.76 ppm) and H^{η} (6.82 ppm). Saturation of the $H^{\gamma 12}$ resonance of Ile-98 (−2.10 ppm) results in a large effect on the other proton of the CH_2 group, $H^{\gamma 11}$ (0.63 ppm). Saturation of the $H^{\beta 1}$ resonance of Met-105 (−0.91 ppm) results in a large effect again on the other proton of the CH_2 group here the $H^{\beta 2}$ (0.44 ppm).

In each case the effects decreased as the length of the saturation pulse was reduced as Figure 2 shows for the saturation of Ile-98 $H^{\gamma 12}$. In Figure 3 the magnitudes of the Overhauser effects are plotted against the time of saturation for the three systems studied, and differences in the rates of development of the effects are apparent. Similar results were obtained at 270 MHz.

When the length of the presaturation pulse (t) is very short, only the irradiated proton (i) has magnetization ($I_z^i(t)$) which differs significantly from the equilibrium magnetization (I_0). Under these conditions, the Overhauser effect ($\eta_j(t)$) on another proton (j) is approximated by eq 1,[7,9,10] where σ_{ij} is the cross-

$$\eta_j(t) = (I_z^j(t) - I_0)/I_0 = \sigma_{ij}t \quad (1)$$

relaxation rate between protons i and j. Cross-relaxation rates between pairs of protons may therefore be determined directly from the initial slope of $\eta_j(t)$ against t. The Overhauser effects obtained with short saturation pulses are small, and the initial slope is hard to define accurately. In order to perform a more accurate analysis, we adopted an approach based on the behavior of two isolated spins. For a two-spin system, the full time dependence of the Overhauser effect is given by eq 2,[1] where ρ_j is

$$\eta_j(t) = (\sigma_{ij}/\rho_j)(1 - e^{-\rho_j t}) \quad (2)$$

the direct relaxation rate of proton j. In a multispin system an expression of this type will be an accurate description of $\eta_j(t)$ provided that no spin k exists which has σ_{ik} large compared with σ_{ij} and also a large value of σ_{jk}. In effect this requires that there is no spin positioned between i and j through which magnetization can be transferred efficiently. The approximation to two-spin behavior will be better at short values of t.

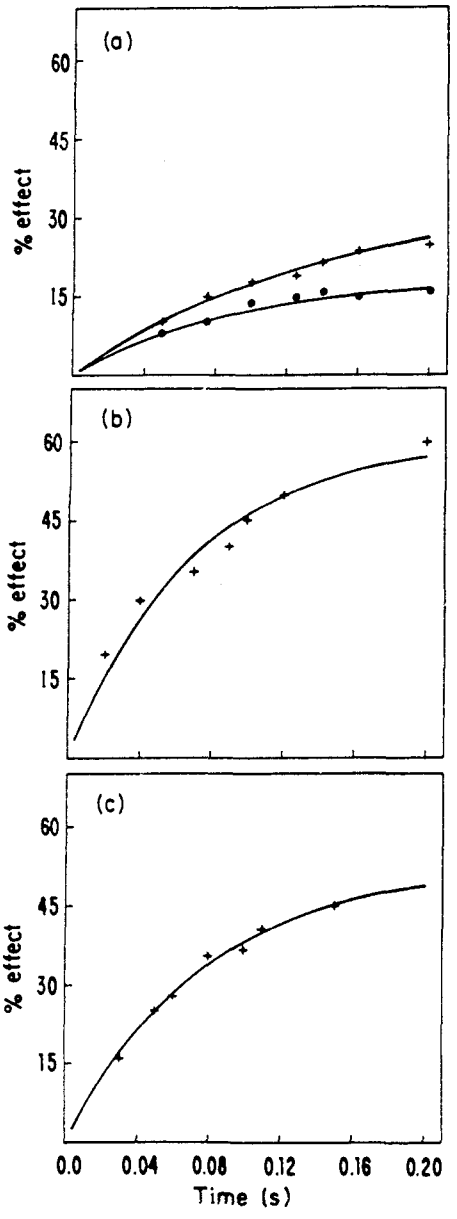

Figure 3. The magnitude of nuclear Overhauser effects at 498 MHz as a function of time of irradiation: (a) $H^{\epsilon 3}$ (upper) and H^{η} (lower) of Trp-28 following irradiation of $H^{\zeta 3}$; (b) $H^{\gamma 11}$ of Ile-98 following irradiation of $H^{\gamma 12}$; (c) $H^{\beta 2}$ of Met-105 following irradiation of $H^{\beta 1}$. The curves are calculated by using the two-spin model as described in the text. Values of σ_{ij} are given in Table I. Values of ρ_j (in s^{-1}) are (a) 6.3 and 7.6, (b) 14.4, and (c) 12.7.

Fits of experimental data to eq 2, treating σ_{ij} and ρ_j as independent parameters, are shown in Figure 3 and provide a good description of the time dependencies. Values of σ_{ij} based on the fits are given in Table I. For given proton pairs, the values obtained at 270 and 498 MHz are closely similar. This is an important result which reduces the probability of systematic experimental errors associated with the characteristics of a given spectrometer.

For dipole–dipole interactions, σ_{ij} and ρ_j can be defined[27] in terms of spectral density functions, J(0), J(ω) and J(2ω), where ω is the resonant frequency. For a two-spin system

$$\sigma_{ij} = (2\pi/5)\gamma^4\hbar^2 \quad (6J(2\omega) - J(0)) \tag{3}$$

$$\rho_j = (2\pi/5)\gamma^4\hbar^2 \quad (J(0) + 3J(\omega) + 6J(2\omega)) \tag{4}$$

$\hbar = h/2\pi$ where h is Planck's constant and γ is the magnetogyric ratio of the proton. In a multispin system, ρ_j becomes a summation over all spins $k \neq j$. The form of the spectral densities is dependent on the model used to describe the motion of the internuclear vector.[13,27-29] One type of motion which must be present is that due to overall tumbling of the protein molecule, and for lysozyme this motion can be described to a good approximation by an isotropic rotational correlation time, τ_R.[30] τ_R for lysozyme has been measured by a variety of different methods, including light scattering,[31,32] fluorescence,[33] and ^{13}C NMR of backbone C^{α} carbons.[30] There is a reasonable agreement between the different methods, and when reduced to 34 °C in D_2O by correcting in the usual way for viscosity and temperature a value of 10 ± 2 ns is obtained. If this type of motion alone determines the relaxation rates, the spectral density functions are of the form[27]

$$J(\omega) = \frac{1}{4\pi r_{ij}^6}\left[\frac{\tau_R}{1 + \omega^2\tau_R^2}\right] \tag{5}$$

where r_{ij} is the distance separating spins i and J. For each of the proton pairs studied in this work r_{ij} is fixed by the geometry of the side-chain residue. Thus, values of σ_{ij} can be calculated on the basis of this model and in Table I are listed values for the three residues studied here. The observed and calculated σ_{ij} values between protons in the Trp-28 ring are, within experimental error, in agreement. The observed values for the proton pairs of Ile-98 and Met-105 are, however, more than a factor of 2 smaller than the calculated values. Before examining the significance of these differences, we must consider possible errors in the two-spin approach.

The three residues studied are in the hydrophobic box region of lysozyme.[34,35] The structure of this part of the molecule in solution has previously been shown to correlate strongly with the crystal structure.[7,18] With use of coordinates based on the crystal structure, and a vaue of τ_R of 10 ns, time dependencies of the Overhauser effects were calculated numerically by the method of Bothner-By and Noggle.[8] The 20 protons closest to each irradiated proton were considered, and values of relaxation rates were calculated on the basis of eq 3–5. These multispin calculations (Figure 4) fit the experimental points for Trp-28 very closely at short times, although they deviate slightly at longer times.[36] The calculated time developments are, however, very

Table I. Cross-Relaxation Rates

residue	proton pair	freq, MHz	σ_{ij}, s^{-1} calcd[a]	σ_{ij}, s^{-1} exptl[b]
Trp-28	$H^{\zeta 3}$-$H^{\epsilon 3}$	270	−2.75 ± 0.54	[−2.5 ± 0.2][c]
		498	−2.76 ± 0.55	−2.5 ± 0.3
Trp-28	$H^{\zeta 3}$-H^{η}	270	−1.90 ± 0.39	[−2.5 ± 0.2][c]
		498	−1.92 ± 0.40	−1.8 ± 0.2
Ile-98	$H^{\gamma 12}$-$H^{\gamma 11}$	270	−18.6 ± 3.4	−6.2 ± 1.1
		498	−18.7 ± 3.4	−8.7 ± 0.9
Met-105	$H^{\beta 1}$-$H^{\beta 2}$	270	−18.6 ± 3.4	−6.4 ± 0.8
		498	−18.7 ± 3.4	−6.8 ± 0.7

[a] Using distances given in Table II, and assuming $\tau_R = 10$ ns. The error limits represent an uncertainty of ±2 ns in τ_R. [b] Derived from the best two-spin fits (see Figure 3). The error limits are determined from the least-squares fitting procedure. [c] The resonances of H^{η} and $H^{\epsilon 3}$ overlap at 270 MHz.

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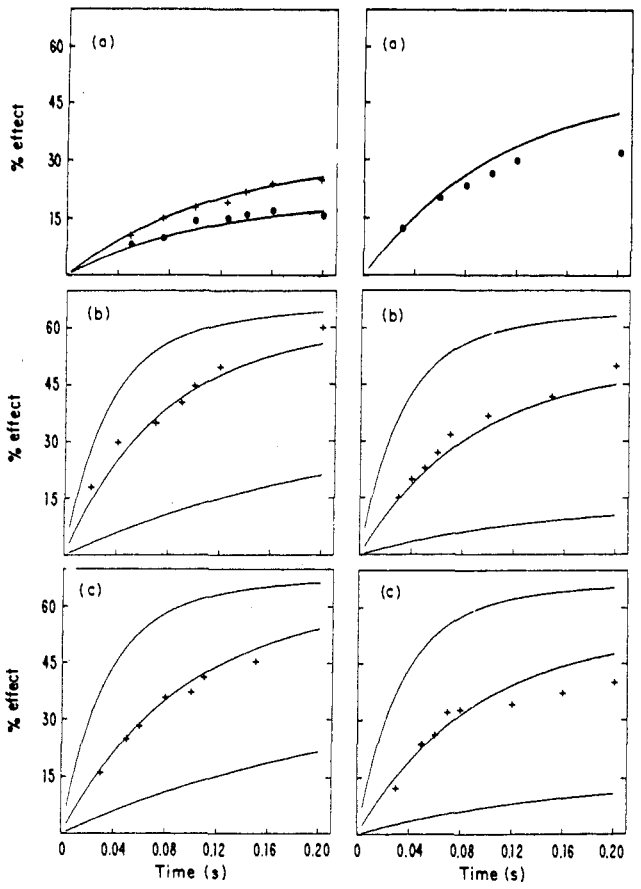

Figure 4. Time dependencies of the nuclear Overhauser effects as in Figure 3 but at 270 MHz (right) as well as at 498 MHz (left). The curves for Trp-28 are calculated by using the multispin model with τ_R = 10 ns as described in the text; at 270 MHz the sum of the effects is plotted as the $H^{\epsilon 3}$ and H^{η} resonances are not resolved. For Ile-98 and Met-105 the top and bottom curves are for τ_R = 10 and 1 ns, respectively. The middle curves are fits to the multispin model having effective correlation times for Ile-98 of 4.5 ns (498 MHz) and 3.3 ns (270 MHz) and for Met-105 of 3.6 ns (498 MHz) and 3.4 ns (270 MHz). These give the values of σ_{ij} listed in Table I.

much faster than those observed experimentally for the CH_2 groups of Ile-98 and Met-105. It is possible, however, to describe well the experimental time developments with the multispin model by using τ_R values significantly lower than 10 ns. Such values may be considered as correlation times which effectively describe the time developments of the Overhauser effect, although no physical significance need be attributed to them. The calculations do indicate, however, that the discrepancies for Ile-98 and Met-105 between calculated σ_{ij} values and those obtained from the two-spin fits cannot be attributed to the approximations of eq 2.[37] Indeed, with use of effective correlation times corresponding to the σ_{ij} values obtained from the two-spin model, see eq 3 and 5, the multispin calculations provide a good description of the time developments (Figure 4) and are very close to the fits obtained from the two-spin model. This is true to a lesser extent for the

(36) In the multispin calculations methyl groups were freely rotating with τ_l = 0.1 ns. This had only a small effect on σ_{ij} and ρ_j values for the protons considered here. Values of ρ_j in s^{-1} obtained from the best fits to the multispin model are (a) for Trp-28, 9.7 and 12.6 (498 MHz) and 13.1 (mean 270 MHz), (b) for Ile 98, 10.4 (498 MHz) and 11.1 (270 MHz), and (c) for Met-105, 11.4 (498 MHz) and 12.1 (270 MHz). These values were found to be extremely sensitive to the crystallographic coordinates. For example, with the less highly refined RS5D coordinates of lysozyme[35] the value for Met-105 $H^{\beta 2}$ differed from that above by more than a factor of 3, because of unreasonably close contact with methyl protons of this residue. This illustrates the difficulty of interpreting relaxation rates between protons whose distances are not fixed by the geometry of the residue in question.

(37) The accuracy of the two-spin approach to interpret the protein system was further tested by calculating the time developments using 20 spins and then fitting to the two-spin model. σ_{ij} and ρ_j values were in very good agreement for the range of correlation times relevant to this work.

Table II. Angular Fluctuations from the Fast Restricted Diffusion Model

residue	proton pair	r^a	$\beta(C^\alpha\text{-}C^\beta)^b$	$\beta(C^\beta\text{-}C^\gamma)^b$	$\theta(C^\alpha\text{-}C^\beta)^c$	$\theta(C^\beta\text{-}C^\gamma)^c$
Trp-28	$H^{\delta 3}$-$H^{\epsilon 3}$	2.43	80	26	0 ± 30	0 ± 30
Trp-28	$H^{\delta 3}$-H^{η}	2.58	57	82	0 ± 30	0 ± 30
Ile-98	$H^{\gamma 12}$-$H^{\gamma 11}$	1.76	124	90	65 ± 17	57 ± 10
Met-105	$H^{\beta 1}$-$H^{\beta 2}$	1.76	90		64 ± 23	

[a] In Å, from ref 39 and 40. [b] Values of β in deg, obtained from the refined crystal structure of lysozyme. β is the angle between the indicated interproton vector and the indicated carbon-carbon bond. [c] θ is rotation in degrees allowed about the indicated bond. τ_R = 10 ns and the average experimental values of σ_{ij} from the 270- and the 498-MHz data were used.

ρ_j values obtained from the fits of Figures 3 and 4. The fits to the experimental data are not very sensitive to the ρ_j values, and correspondingly the experimental estimates of ρ_j are subject to much larger errors than values of σ_{ij}.[23] Indeed ρ_j values such as in Figure 3 are here considered only as parameters in the fits to the two-spin model and not as true relaxation rates. The calculated ρ_j values are also subject to much larger errors than the σ_{ij} values. For the proton pairs considered here, between 37% and 80% of the contribution to ρ_j arises from protons whose distances to proton j are not independent of the protein conformation. Errors of only tenths of ångströms in the coordinates have significant effects on ρ_j.[36] In addition the effects of internal motions on ρ_j are much more complex than on σ_{ij} (see below) because of the number of spin pairs involved. For these reasons the significance of ρ_j values is not discussed further.

Internal Motion. The differences between the values of σ_{ij} observed experimentally and those expected for a rigid protein could arise because of an incorrect assumption of 1.76 Å for the separation of the two protons of a CH_2 group. In order, however, for a correlation time of 10 ns to describe the cross-relaxation rates for the proton pairs of Ile-98 and Met-105, the separation would need to be close to 2.05 Å. This is very much larger than reported values,[25,38,39] which are in the range 1.75–1.79 Å, and cannot be considered chemically reasonable. Thus, while overall molecular tumbling appears to be adequate to describe the motion of Trp-28, additional motion must be invoked to explain the cross-relaxation rates for the CH_2 groups of the two aliphatic residues.

The possible internal motions of a CH_2 group in a protein are complex. Nevertheless, a qualitive interpretation of NMR data using simple motional models has proved useful in investigating specific types of molecular motions in other systems.[13-16,29] Recent theoretical studies of protein dynamics have shown that fluctuations of torsional bond angles can occur on a picosecond timescale.[19-21] As an example of the effects of fast internal motions on cross-relaxation rates σ_{ij} values have been calculated by using a model which describes the motions as restricted rotations about defined side-chain bonds. In the fast motional limit, where the internal fluctuations are much faster than overall tumbling and the resonant frequencies, the spectral densities can be approximated by expressions of the form[13]

$$J(\omega) = \left[\left(\frac{3\cos^2\beta - 1}{2}\right)^2 + 3\sin^2\beta\cos^2\beta\left(\frac{\sin\theta}{\theta}\right)^2 + \tfrac{3}{4}\sin^4\beta\left(\frac{\sin 2\theta}{2\theta}\right)^2\right]\frac{\tau_R}{1+\omega^2\tau_R^2} \quad (6)$$

For the system studied here, the fast motional limit will be a good approximation if the internal correlation time $\tau_l \lesssim 10^{-10}$ s. β is

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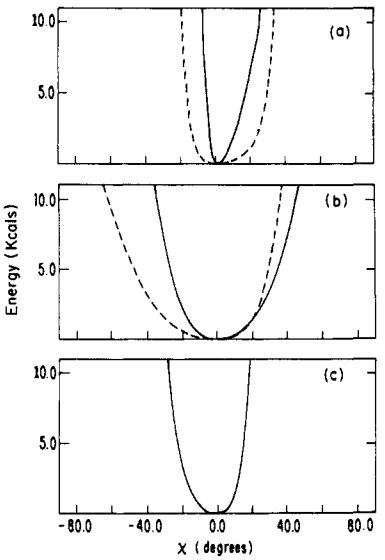

Figure 5. Conformational energy as a function of torsion angle (χ) for (a) Trp-28, (b) Ile-98, and (c) Met-105. Rotations about C^{α}–C^{β} bonds are represented by solid lines and about C^{β}–C^{γ} bonds by broken lines.

the angle which the vector joining the proton pair makes with the axis of bond rotation, and θ is the angle through which rotation occurs. Because the angle β will depend on which bond is being considered, it is possible in principle to determine the effect that rotation about a variety of bonds will have. In Table II are listed the angles β which the various interproton vectors make with the axis of rotation and values of θ obtained by using the fast rotation model and the experimental cross-relaxation rates. For Trp-28 no significant fluctuations about either C^{α}–C^{β} or C^{β}–C^{γ} bonds are indicated, although experimental errors in both the overall correlation time and in the cross-relaxation rates cannot exclude fluctuations of less than 30°. For Met-105 the model requires that $\theta = 64° \pm 23°$ for fluctuations about the C^{α}–C^{β} bond and Ile-98 $\theta = 65° \pm 17°$ and $57° \pm 10°$ for fluctuations about the C^{α}–C^{β} and C^{β}–C^{γ} bonds, respectively, taken separately.

Rotations about individual bonds in a protein will cause close contacts between the rotated atoms and neighboring atoms. These close contacts will give rise to large van der Waals interactions which will produce unfavorable barriers for such motions.[26] These barriers will constrain both the range of allowable rotation and the frequency at which these rotations occur. In order to investigate the energetic consequences of rotation about the side chain bonds of the three residues, we calculated the conformational energy of lysozyme as a function of the various rotation angles. The results of these calculations (Figure 5) are that the orientation of each residue in the crystal structure corresponds to a minimum energy and that in each case large steep barriers exist to free rotation about any of the bonds in question. The energy required for a given rotation differs for the three residues being less steep near the minium for Ile-98 than for Met-105 and Trp-28.

The experimental results for Met-105 and Ile-98 require angular fluctuations which are larger than those expected from the conformational energy calculations to be energetically feasible on a subnanosecond time scale.[20] The energy barriers found in the rigid crystal structure are decreased by concerted motions of the different residues. These have been shown to occur in theoretical studies of the bovine pancreatic trypsin inhibitor (BPTI) and other protein systems.[20,26] The experimental results for Trp-28 might suggest that the reduction of the barriers is more difficult for the rigid tryptophan residue than for the smaller more flexible aliphatic residues.

Discussion

Accurate cross-relaxation rates between pairs of protons of three residues in lysozyme have been obtained from the time dependence of nuclear Overhauser effects by using a simple two-spin analysis. The method developed is a general one, provided that experiments are carried out to ensure effectively instantaneous saturation and that calculations are performed to establish the validity of the two-spin approximation. Cross-relaxation rates have been calculated from a model of rigid molecular rotation. The ratios of the observed and calculated rates for the proton pairs have been found to vary by more than a factor of 2, even though all the residues are in the same region of the protein structure. Similarly, when a isotropic tumbling model was used to fit the experimental data, the resulting effective correlation times varied by more than a factor of 2.

The results show first that different degrees of internal motion occur for the residues studied. Cross-relaxation rates of the type measured in this work cannot define directly both the amplitudes and correlation times of internal motions, but they can be used to examine specific types of motion proposed to exist in a protein. As an example, rapid fluctuations of bond torsional angles of the type predicted in simulations of protein dynamics[19-21] have been studied here by using a simple restricted rotation model.[13] For the Trp-28 residue no internal motion is indicated, although fluctuations of up to ±30° about either the C^{α}–C^{β} or C^{β}–C^{γ} bond would be within the experimental errors since cross-relaxation rates are rather insensitive to small angle fluctuations.[13] The rather restricted nature of Trp-28 is consistent with fluorescence depolarization studies of tryptophans in a number of proteins;[41] the largest tryptophan fluctuations on a sub-nanosecond timescale found[41] by this technique were less than 35°. For the aliphatic residues Ile-98 and Met-105 the existence of extensive internal motion is quite clear. For both these residues fluctuations equivalent to rotations of about ±60° about individual side-chain bonds are required, on the fast rotation model, to produce the observed relaxation rates. These fluctuations are large enough to accommodate the torsion angle variations observed on subpicosecond time scales in theoretical simulations of protein dynamics[21] but could also represent the effects of smaller angular fluctuations about a number of different bonds. Experimental data are also available for other proteins to compare with these results. An example for direct comparison concerns isoleucine residues in myoglobin that have been shown by ^{13}C NMR relaxation studies to be undergoing different degrees of fast motion about C^{α}–C^{β} and C^{β}–C^{γ} bonds.[14] Angular fluctuations with picosecond correlation times were found to be in the range of ±30° to ±50°. More general evidence for internal motions in a variety of proteins has been summarized in recent reviews.[21,42] Some insight into the nature of internal motions in a protein can be gained by consideration of the energies involved in rotating the isoleucine and methionine residues in the lysozyme structure through the angular ranges determined from the NMR results. Calculations using a rigid rotation model support the concept that motions in the protein are concerted or cooperative. The small mobility of Trp-28 compared with Ile-98 or Met-105 is attributed primarily to the large size and rigidity of the aromatic ring rather than to a very different environment in the structure.

Motions other than these fast fluctuations are by no means eliminated by the experimental results. The magnitudes of the fast fluctuations are maximum values consistent with the data and would be reduced if additional slower motions were also present. In the absence of the fast motions, however, any slower motions must involve greater angular fluctuations. As an example, even free rotation about a given side-chain bond would be insufficient to explain the cross-relaxation data if it took place with a correlation time longer than about 5×10^{-8} s.[43] Additional studies are, however, required to define more fully the internal dynamics of the protein. One approach to further interpretation of cross-relaxation data is to combine the nuclear Overhauser

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(43) With use of a model of free internal rotation,[28] the values of τ_j required to give the expected σ_{ij} values are approximately 2×10^{-8} s for both Ile-98 and Met-105. The energy calculations, however, indicate that the barriers to free rotation are extremely high.

method with other measurements of relaxation phenomena. The results in this paper show that side-chain internal motions in proteins have timescales and amplitudes which are sufficient to affect longitudinal relaxation rates. This implies that previous models[17] which consider that the only motions of significance for proton relaxation are overall tumbling and methyl group rotations are too simple. Preliminary results[16,44] have shown that data from inversion recovery and T_2 measurements at different frequencies can be interpreted in terms of a simple model of more general internal motion. Another approach is to use the σ_{ij} values in conjunction with calculations of protein dynamics. Recently, correlation functions have been calculated directly from the protein dynamics simulations.[45-47] From these correlation functions it has been predicted that ^{13}C spin-lattice relaxation times are simply changed by scaling factors from those expected for a rigid molecule. These scaling factors are directly analogous to the ratios of observed and calculated σ_{ij} values derived in the present studies.

Another important consequence of differences between observed σ_{ij} values and those calculated on the basis of a rigid model of the protein (i.e., of a variation in effective correlation times of different residues) relates to structural studies of proteins by using proton Overhauser effects. These studies have previously assumed[7] that a single correlation time describes the motion of all proton pairs. The nuclear Overhauser effect depends directly on the value of the correlation time as well as on the inverse sixth power of the distances between protons. If distances between the proton pairs were, however, to be calculated from Overhasuer effects, variations of even a factor of 3 in the scaling factor for σ_{ij} values would result in errors of at most 20% in relative distances. Because distances measured by this technique are generally 5 Å or less,[7] the errors resulting directly from neglect of motional effects of the type considered here are likely to be less than ±0.5 Å. These errors are not large when compared with those of even the highest resolution protein crystal structures and are unlikely to be the dominant errors in NMR distance measurements except at the very highest level of experimental accuracy. Distances measured from the cross-relaxation rates between protons whose separation is not motionally invariant will, however, be averaged over the various interconverting states. That the averaged distances obtained from nuclear Overhauser effects in proteins may not be greatly different from the distances between the average coordinates can be deduced[7] from the high level of correlation between Overhauser effects and distances derived from the crystallographic coordinates of lysozyme. The present results give confidence in the use of proton Overhauser effects for defining accurate structural and dynamical features of proteins in solution.

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Structure Revision of the Antibiotic Vancomycin. The Use of Nuclear Overhauser Effect Difference Spectroscopy

Michael P. Williamson and Dudley H. Williams*

Contribution from the University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, United Kingdom. *Received March 26, 1981*

Abstract: Through the use of nuclear Overhauser effect difference spectroscopy (nOeds), the negative nOe has a great potential for structure elucidation of relatively large organic molecules. This potential could be compromised by even limited spin diffusion. The antibiotic vancomycin has been examined by the nOeds technique to determine the amount of structural and stereochemical information which can be derived. Recognizing, and making allowances for, spin diffusion and "spin migration" (chemical exchange of perturbed NH and OH proton spins), the relative stereochemistry at the majority of the chiral centers of the aglycon portion can be derived. The power of the method is illustrated by the fact that the data demand a significant revision of the structure of vancomycin (previously based on an X-ray study of a crystalline degradation product, CDPI). Where spin diffusion is not involved, the interproton distances calculated from nOe's are normally in good agreement with those from X-ray data. Two exceptions are shown to indicate the oscillation of an aromatic ring in the antibiotic.

A nuclear Overhauser effect (nOe) is observed when irradiation of a proton causes a change in the integrated intensity of the resonance of a second proton. This second proton must be predominantly dipole-dipole coupled to the irradiated proton and relatively close in space to it. The rate of buildup of the nOe shows an r^{-6} dependence.[1] If the integrated intensity of the observed proton increases (positive nOe), information on relative interproton distances can normally be obtained; such is the case for molecules which have a relatively small rotational correlation time τ_c ($\omega\tau_c < 1$). However, when τ_c is relatively large ($\omega\tau_c > 1$), the nOe becomes negative. Under these circumstances, spin-lattice relaxation may become relatively inefficient (especially if $\omega\tau_c \gg 1$), and the perturbations of spin populations caused by the irradiating field pass from spin to spin; the nOe is said to spread by spin diffusion.[2-5] Spin diffusion is more noticeable if the

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