Studies of β-Sheet Structure in Lysozyme by Proton Nuclear Magnetic Resonance. Assignments and Analysis of Spin–Spin Coupling Constants†

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ABSTRACT: Resonances of H^{α}, H^{β}, and H^{N} (amide) protons have been assigned in the NMR spectrum for ten residues in a region of β-sheet structure of lysozyme. The assignments were achieved primarily by interpretation of nuclear Overhauser effects in conjunction with spin decoupling. The H^{N} hydrogens involved in main-chain hydrogen bonding were found to exchange slowly with D_2O solvent, although one of the most slowly exchanging H^{N} hydrogens is not classified as being involved in a hydrogen bond in the crystal structure. Spin–spin coupling constants between H^{α} protons and H^{N} and H^{β} protons correlated well with values predicted from the crystal structure by means of the Karplus relationship. For no residues are the coupling constant discrepancies greater than 2.5 Hz. This indicates that for the residues studied here the torsion angles ϕ and χ_1 defined in the crystal structure describe accurately, generally well within 20°, those for the average solution state.

Proton nuclear magnetic resonance (NMR) studies of lysozyme, a protein containing 129 amino acid residues, have resulted in the assignment of many resonances of side-chain aromatic protons and methyl groups [summarized in Poulsen et al. (1980)]. With these assignments, information about the static and dynamic aspects of the protein in solution has been acquired (Campbell et al., 1975; Dobson, 1977; Blake et al., 1978; Perkins & Dwek, 1980; Poulsen et al., 1980) particularly by comparison with results from X-ray diffraction studies of lysozyme in crystals (Blake et al., 1965, 1967; Sternberg et al., 1979; Grace, 1980). Very few resonances of the main-chain H^{α} and H^{N} protons have previously been identified because of the problems of overlapping resonances and of distinguishing between the chemically similar groups of the different residues. In the present work, assignment of most of the H^{α}, H^{β}, and H^{N} resonances of ten residues of lysozyme has been achieved. These residues are all between 43 and 65 in the amino acid sequence and are in a region of the molecule having a mainly β-pleated sheet structure and which forms a part of the active-site cleft (Blake et al., 1967). The assignment method is based on the use of nuclear Overhauser effects (Noggle & Schirmer, 1971); it has been found previously for lysozyme that the effects are very strongly correlated with internuclear distances derived from the crystal structure (Poulsen et al., 1980; Dobson et al., 1980; Delepierre et al., 1981). Similar methods have proved successful in the assignment of proton resonances in the NMR spectra of other proteins, for example, the bovine pancreatic trypsin inhibitor protein (Dubs et al., 1979; Wagner & Wüthrich, 1979), cytochrome *c* (Moore & Williams, 1980), and a neurotoxin (Inagaki et al., 1982).

The assignment of H^{α}, H^{β}, and H^{N} resonances is of importance in characterizing the protein structure and dynamics in solution. In particular, as has been shown clearly for amino acids and peptides, the spin–spin coupling constants provide detailed information about the ϕ and χ_1 torsion angles for individual residues (Feeney, 1975; Bystrov, 1976; DeMarco et al., 1978; Nagayama, 1981). In addition, if the solvent exchange rates of individual H^{N} hydrogens can be measured, information concerning the mechanisms of hydrogen exchange and hence the dynamics of the protein structure can be obtained (Woodward & Hilton, 1979; Wagner & Wüthrich, 1979; Wedin et al., 1982). Some initial results for the β-sheet region of lysozyme are presented here.

Experimental Procedures

Lysozyme from hen egg white (EC 3.2.1.17) was obtained from Sigma Chemical Co. and dialyzed extensively at pH 3.0 before use. NMR samples were 5 mM in lysozyme at pH 3.8 in D_2O unless otherwise stated. Before NMR spectra were recorded, samples were typically equilibrated in 99.8% D_2O for at least 2 h at the temperature of the experiment, in order to permit the most labile hydrogens to exchange with the deuterium of the solvent. In certain cases all exchangeable hydrogens were replaced with deuterium by reversible thermal denaturation in D_2O. Chemical shift values are quoted for spectra at pH 3.8 and 57 °C 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 dioxane. Coupling constants were measured by observing H^{α} and H^{N} resonances either directly in the spectra or in Overhauser difference spectra as illustrated below. In certain cases, confirmation of the values was obtained from spectral simulations. Hydrogen exchange rates were estimated by measuring changes in peak heights of H^{N} resonances as a function of time after dissolution in D_2O under defined conditions. The existence of a proton or deuteron on peptide nitrogen atoms was also investigated by observing the coupling pattern of the H^{α} resonances.

^1H NMR spectra were recorded at 300 MHz on a Bruker WH 300 spectrometer and at 470 MHz on the home-built spectrometer of the Oxford Enzyme Group, equipped with an

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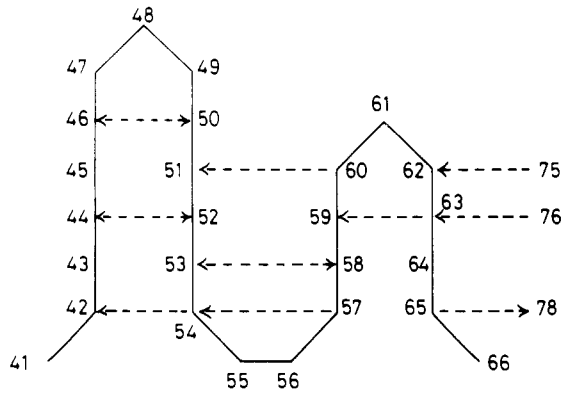

FIGURE 1: Schematic illustration of the structure of lysozyme from residues 41–66. Main-chain hydrogen bonds from NH to CO are shown by arrows. Based on diagrams by Imoto et al. (1972) and Grace (1980).

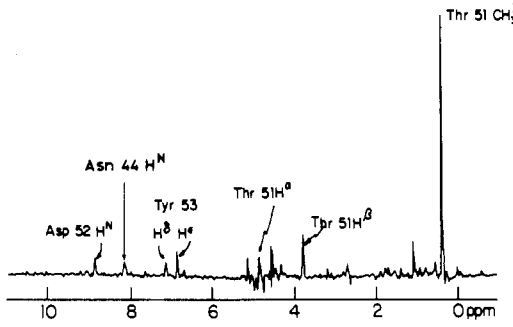

FIGURE 2: 470-MHz difference spectrum at 37 °C of 6 mM lysozyme, freshly dissolved in D_2O at pH 2.9, following saturation for 1.5 s of the CH_3 resonance of Thr-51. The assignments are discussed in the text.

Oxford Instrument Co. magnet. Spectra were also obtained at the NMR Facility for Biomolecular Research, Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, at 270 MHz on a Bruker spectrometer and at 500 MHz on the home-built spectrometer of the NMR Facility. The methods used for resolution enhancement (employed in all spectra shown in this paper) and for detection of spin-decoupling and nuclear Overhauser effects have previously been described fully (Campbell & Dobson, 1979; Poulsen et al., 1980). Proton coordinates and internuclear distances based on the X-ray structure of lysozyme (Grace, 1980) were obtained as described previously (Poulsen et al., 1980).

Results and Discussion

Assignments. The assignment of resonances of main-chain protons in the β-sheet region of lysozyme (Figure 1) was initiated from the previous assignments of the resonances of side-chain protons of Thr-51, Tyr-53, and Trp-63 (Campbell et al., 1975; Cassels et al., 1978). It has been shown previously for lysozyme that nuclear Overhauser effects resulting from saturation of resonances of individual protons correlate very strongly with distances based on the X-ray crystal structure (Poulsen et al., 1980; Dobson et al., 1980). Overhauser effects under the conditions described were found to be very small between protons more than 5 Å apart and to be large only between protons closer together than about 3 Å. The first step in the assignment process was therefore to saturate in turn the resonances of the CH_3^{γ} of Thr-51, the $H^{\epsilon 1,2}$ of Tyr-53, and the $H^{\delta 1}$ of Trp-63. The difference spectra, one of which is shown in Figure 2, revealed many well-resolved resonances from peptide H^N protons and from resonances in the H^{α} region of the spectrum, along with others in the more crowded spectral

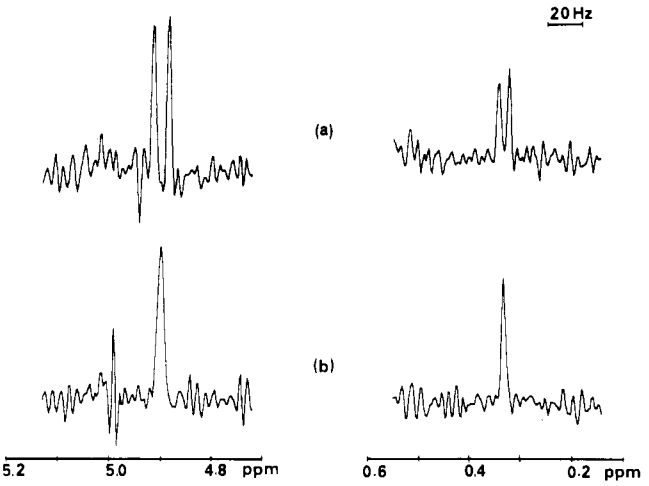

FIGURE 3: Assignment of resonances using a combination of Overhauser and spin-decoupling experiments at 300 MHz. (a) Part of the difference spectrum resulting from the saturation for 1.5 s of an H^N resonance, at 8.82 ppm, showing large Overhauser effects on the resonances of an H^{α} proton (left) and a CH_3 group (right). The spin–spin coupling constants for these resonances are clearly revealed. (b) The same experiment carried out while applying selective irradiation, during acquisition, at 3.75 ppm. The decoupling of both resonances is observed and indicates that they and the resonance at 3.75 ppm arise from the same residue. The CH_3 group has previously been assigned to Thr-51, permitting the assignment of the H^{α} and H^{β} resonance to be made. The singlet nature of the H^{α} resonance in (b) indicates that under these conditions (57 °C, pH 3.8 in D_2O, after several hours) the H^N of this residue has exchanged with D_2O. The H^N resonance at 8.82 ppm is of a different residue, close to Thr-51. The final assignment of this resonance is to Asp-52.

region between 1 and 4.5 ppm. It was assumed all these resonances were candidates for assignments to residues in the β-sheet region.

The next step was to use spin decoupling to relate resonances of the same residue to each other. Again, difference spectroscopy was used extensively, and clear decoupling effects could be observed in almost all cases between H^{α}, H^N, and H^{β} resonances. Because of the overlap of many resonances, the experiments to observe both Overhauser and spin-decoupling effects were repeated under a variety of conditions. For example, changes in temperature between 37 and 62 °C and of pH between 2 and 6 resulted in many small shifts which permitted ambiguities to be resolved. Larger shifts were produced by addition of paramagnetic lanthanide ions, which bind to lysozyme as discussed previously (Campbell et al., 1975; Dobson & Williams, 1977). Additional extensive Overhauser experiments were then carried out by selective saturation of the various H^{α} and H^N resonances under conditions where resolution was highest (Figure 3). In cases where even these experiments did not permit unambiguous identification of specific resonances, combined spin-decoupling and Overhauser experiments were performed (Figure 3). Comparison of experiments carried out by using lysozyme reversibly denatured in D_2O with experiments carried out by using lysozyme simply dissolved in D_2O for different periods of time permitted the identification of residues with slowly exchanging H^N hydrogens to be made.

Examination of the coupling patterns for the various spin systems permits limited information about the type of residue to be obtained. For example, the detection that two H^{β} protons are coupled to an H^{α} proton eliminates the possibility that the residue is alanine, glycine, isoleucine, threonine, or valine. The further analysis of coupling patterns, and of chemical shift values, could provide further information to suggest the nature of the residue. In fact this information was not used at this

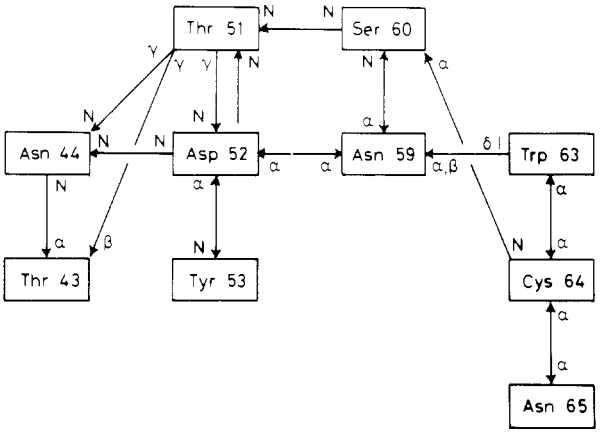

FIGURE 4: Schematic illustration of the major nuclear Overhauser effects used in the assignment procedure. Residues are represented by boxes, and the arrows point from the residue whose resonance was saturated to the residue on which an Overhauser effect was observed; the specific protons involved are indicated in each case.

Table I: Chemical Shift Values of Assigned Resonances

residue	chemical shift[a] H^N	H^α	H^β	$H^{\beta'}$	H^γ
Thr-43		5.14	3.79		1.06
Asn-44	8.16	5.04	(2.99)	2.74	
Thr-51	9.04	4.90	3.75		0.33
Asp-52	8.82	5.22	2.63	(2.02)	
Tyr-53	9.05	4.77	(2.95)	(2.67)	
Asn-59		5.66	3.32	3.03	
Ser-60	9.20	5.18	4.40	(4.40)	
Trp-63		4.97	3.50[b]	3.50[b]	
Cys-64	7.62	5.82	3.05	2.53	
Asn-65	8.25	5.52	2.89	2.46	

[a] In ppm, for lysozyme at pH 3.8, 55 °C. [b] These resonances are broad.

stage but merely provided confidence in the final conclusions.

It was next assumed that large Overhauser effects ($\gtrsim$5% under the conditions used here; see Figure 2) could only arise between protons whose distance apart in the crystal structure was less than 6 Å. On the basis of previous data (Poulsen et al., 1980; Delepierre et al., 1981), this is a conservative assumption but was sufficient for the present problem. Distances between protons in the crystal structure were calculated, and the Overhauser experiments were examined in the light of these distances. The possible assignment schemes were considered, and one, that shown schematically in Figure 4, was consistent with all the data. This illustrates that when sufficient pairs of protons are linked by Overhauser effects, only very safe assumptions about the distance dependence need be made. The assignments of the ten residues made in this work are listed in Table I. Other assignments have been made by using these methods but are not discussed at present. It is notable that the rather complex nature of the β-sheet region of lysozyme necessitated the detailed consideration of much experimental data.

Although no Overhauser or spin-decoupling effects have been detected that are inconsistent with these assignments, there are expected effects which have not yet been observed unambiguously. These involve only effects on protons whose resonances have not been detected. Most notable of these concern resonances of Trp-63, and the reason here is likely to be related to the observation that the resonances of this residue are characteristically broad (Cassels et al., 1978; C. M. Dobson, F. M. Poulsen, and C. Redfield, unpublished results); the position of the H^N resonance is as yet uncertain. The resonances of Ile-58 have not yet been observed unambiguously, probably because of the degree of overlap in the region of the spectra where the H^α resonance is suspected to lie.

Structural Correlations. The chemical shift values for the assigned protons given in Table I may be compared with those observed for amino acid residues in denatured proteins or small peptides (McDonald & Phillips, 1969; Bundi & Wüthrich, 1979) and the differences attributed to the effects of the local environment produced by protein folding. These observed secondary shifts exceed 1 ppm in only one case (−1.13 ppm for H^α of Cys-64) and are generally of the same order as observed previously for other lysozyme resonances (Campbell et al., 1975; Dobson, 1977; Perkins & Dwek, 1980). The shifts contain contributions from the effects of ring currents, of other local anisotropies of the magnetic susceptibility, and of electric fields (Sternlicht & Wilson, 1967). Ring-current shifts were calculated for the protons assigned in this work by using methods described previously (Sternlicht & Wilson, 1967; McDonald & Phillips, 1969; Perkins & Dwek, 1980) but were much smaller than the observed secondary shifts, rarely exceeding ±0.2 ppm. Clearly factors other than ring-current shifts are dominant here, but calculation methods are not yet well established for these. It is notable that all the H^α resonances observed are shifted to low field by the protein folding and that the majority of the lowest field H^α resonances in lysozyme belong to the β-sheet region of the structure (Figure 5).

The chemical shifts of several of the assigned resonances have been observed to be pH dependent, in particular the H^α and H^β resonances of Asp-52 and the H^α resonances of Asn-65. The effects on the former resonances may be attributed to the ionization of Asp-52 itself and are similar in magnitude to those observed for aspartic acid itself (Feeney et al., 1972). From these shifts the pK value of Asp-52 was observed to be 3.7, in good agreement with previous estimates (Rupley et al., 1974; Dobson & Williams, 1977). The effect on the Asn-65 is attributed to the ionization of Asp-66, and the low pK value ($\lesssim$2.0) is consistent with predictions from crystallographic studies (Imoto et al., 1972). It is perhaps surprising that no significant shifts with pH are observed on side-chain resonances of Tyr-53, for in the crystal structure the carboxylic acid group of Asp-66 is hydrogen bonded to the hydroxyl group of Tyr-53 (Imoto et al., 1972).

Of the residues with assigned resonances in the β-sheet region, the H^N hydrogens of Thr-43 and Asn-59 exchange rapidly with solvent ($t_{1/2} \lesssim 30$ s at pH 3.8, 37 °C) compared with the others. This is consistent with the exposure of these hydrogens to solvent and their lack of involvement in internal hydrogen bonding, as observed in the crystal structure (Imoto et al., 1972). Of the H^N hydrogens observed to exchange more slowly ($t_{1/2} \gtrsim 50$ h), those of Asn-44, Asp-52, Tyr-53, Ser-60, and Asn-65 are all completely buried from solvent (M. J. E. Sternberg, personal communication; Lee & Richards, 1971; Richmond & Richards, 1978) and involved in NH–CO main-chain hydrogen bonds (Figure 1) intrinsic to the β-sheet structure. The H^N hydrogen of Cys-64, however, exchanges at least as slowly as these and while buried is not involved in a defined hydrogen bond in the crystal structure although it is directed toward the carbonyl group of Asn-59 (Grace, 1980). Further studies are in progress to investigate the exchange kinetics in detail.

For most of the assigned H^α and H^N resonances, it has proved possible to observe and analyze the spin–spin coupling patterns to give the three-bond coupling constants $^3J_{HNC^\alpha H}$ and

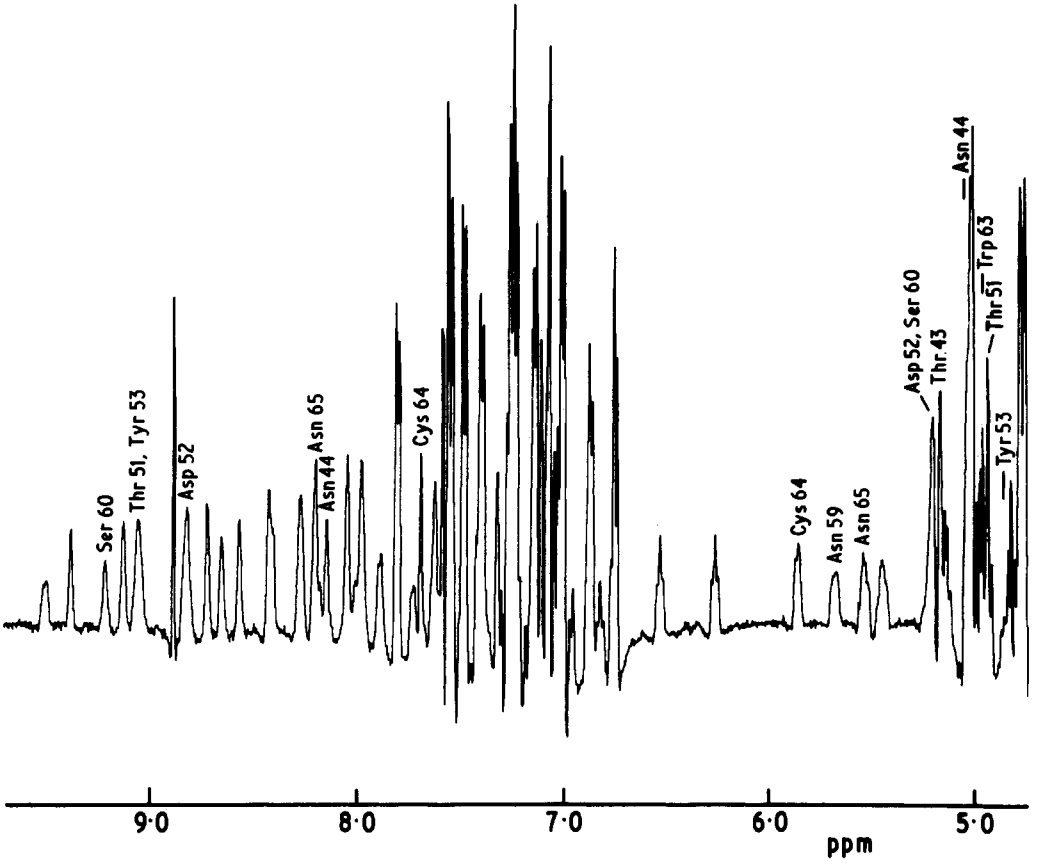

FIGURE 5: Low-field region of the 470-MHz spectrum of 6 mM lysozyme at 37 °C, pH 3.8. The assignments of H^N and H^α resonances are indicated.

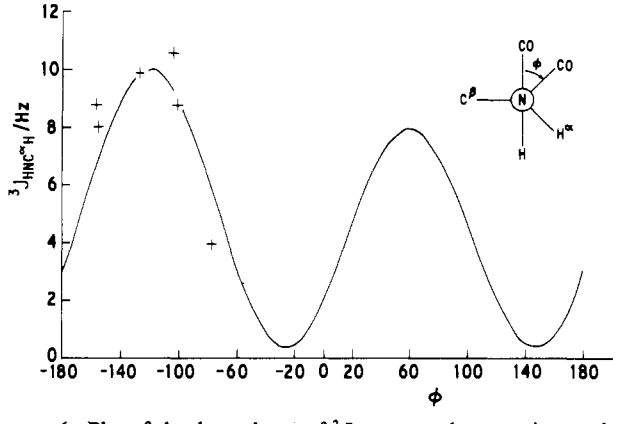

FIGURE 6: Plot of the dependence of $^3J_{HNC^\alpha H}$ on the ϕ torsion angle, using the relationship $^3J_{HNC^\alpha H} = (9.8 \cos^2 \theta - 1.1 \cos \theta + 0.4 \sin^2 \theta)/1.09$. The crosses represent observed coupling constants and values of ϕ defined in the crystal structure (see Table II).

$^3J_{HC^\alpha C^\beta H}$ with an accuracy of about ±1 Hz (Table II). These are related to the ϕ and χ_1 torsion angles, respectively, and the form of the relationships based on the work of Karplus (1959, 1963) has been discussed in detail by Bystrov (1976). The values of coupling constants expected for the torsion angles defined in the crystal structure were calculated by using expressions of the form

$$^3J = A \cos^2 \theta - B \cos \theta + C \sin^2 \theta$$

where θ is the H–X–C–H dihedral angle (see Figures 6 and 7). For $^3J_{HNC^\alpha H}$, the values of A, B, and C were taken to be 9.8, 1.1, and 0.4, respectively, but the calculated coupling constants were then divided by 1.09 to allow for electronegativity effects (Bystrov, 1976). For $^3J_{HC^\alpha C^\beta H}$ the corresponding values were taken to be 11.0, 1.4, and 1.6 (Kopple et al., 1973).

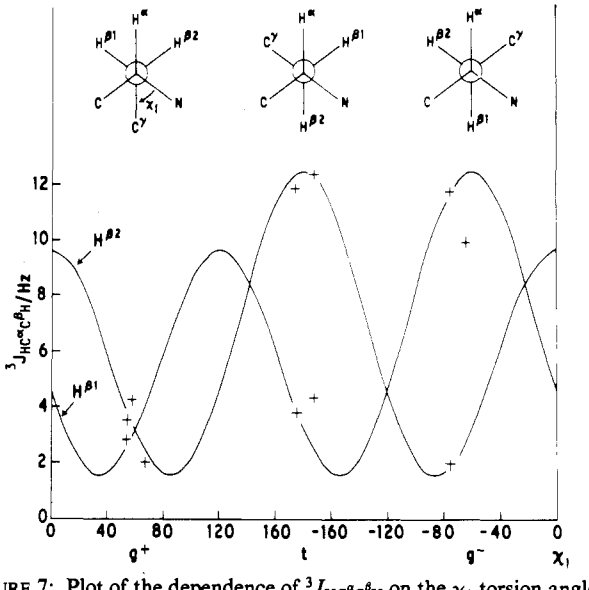

FIGURE 7: Plot of the dependence of $^3J_{HC^\alpha C^\beta H}$ on the χ_1 torsion angle, using the relationship $^3J_{HC^\alpha C^\beta H} = 11.0 \cos^2 \theta - 1.4 \cos \theta + 1.6 \sin^2 \theta$. The crosses represent observed coupling constants and values of ϕ defined in the crystal structure (see Table II), except for the threonine residues where the plotted coupling constants are 1.08 times the observed values (see text).

For the case of threonine only, the calculated coupling constants were divided by 1.08 to allow for the presence of the hydroxyl group at the C^β position (Kopple et al., 1973). These calculations are expected, on the basis of previous work, to be accurate to better than ±1 Hz (Bystrov, 1976).

The data of Table II, plotted in Figures 6 and 7, demonstrate a rather close correlation between the observed coupling

Table II: Observed[a] and Calculated[b] Coupling Constants

residue	ϕ[c]	χ_1[c]	rotamer[c]	$^3J_{HNC^\alpha H}$ obsd	$^3J_{HNC^\alpha H}$ calcd	$^3J_{HC^\alpha C^\beta H}$ obsd[d]	$^3J_{HC^\alpha C^\beta H}$ calcd[d]
Thr-43	−131.4	57.9	g^+		9.6	3.9[e]	3.2
Asn-44	−156.9	−79.1	g^-	8.7	6.7		11.3
							1.7
Thr-51	−142.9	−62.3	g^-		8.6	9.1[e]	11.4
Asp-52	−101.5	−75.3	g^-	8.8	8.9	11.7	11.7
						2 ± 2[f]	1.9
Tyr-53	−126.9	−67.9	g^-	9.8	9.8		12.2
							2.4
Asn-59	−83.6	−172.5	t		6.8	12.2	12.2
						4.3	2.4
Ser-60	−76.7	67.0	g^+	3.9	5.6	2 ± 2[f]	4.2
						2 ± 2[f]	2.5
Trp-63	−108.9	−51.6	g^-		9.6		12.2
							4.4
Cys-64	−156.0	54.4	g^+	8.0	6.8	3.4	4.0
						2.4	2.6
Asn-65	−103.0	174.9	t	10.7	9.2	11.7	12.3
						3.8	3.9

[a] Values in Hz; error approximately ±1 Hz, for lysozyme at pH 3.8, 57 °C. [b] See text. It is interesting that the highly refined coordinates used here (Grace, 1980) give calculated coupling constants in somewhat better agreement with experiment than do the less highly refined coordinates given in Imoto et al. (1972). [c] From the crystal structure. See Figure 7 for definition of rotamers. [d] Distinction between H^{β_1} and H^{β_2} could not be made experimentally without ambiguity. Where two observed or calculated values are given for a residue, the larger is given first in each case. [e] For both threonine residues, $^3J_{HC^\beta C^\gamma H}$ was observed to be 6.8 Hz, in close agreement to values found for the amino acid, 6.7 Hz (Roberts & Jardetzky, 1970). [f] No observable coupling exists; this indicates that the coupling constants are less than 4 Hz.

constants and those calculated as described above. For $^3J_{HNC^\alpha H}$ there is agreement within 2 Hz, that is within the limitations of the experimental observations and theoretical treatment, for all residues. This implies (see Figure 6) that for these residues the torsion angles describing the average solution structure are very well described by those derived in the crystallographic analysis.

With the exception of only one residue, Thr-51, the observed $^3J_{HC^\alpha C^\beta H}$ values are either less than 5 Hz or more than 11 Hz. Further, for those residues with two H^β protons, either one coupling constant is large and the other is small, or both are small. This shows for the solution structure the preference of the side chains for staggered conformations (Figure 7). Similar conclusions for proteins in crystals have been drawn from analysis of the structures of many proteins (Chandrasekara & Ramachandran, 1970; Janin et al., 1978; Bhat et al., 1979; Dezube et al., 1981) and from theoretical calculations (Gelin & Karplus, 1975, 1979). The level of agreement between the observed and calculated coupling constants (Table II and Figure 7) suggests further that the predominant rotamer existing in solution corresponds to that existing in the crystal for each of the residues considered here and that populations of other rotameric states are small. The discrepancies between observed and calculated coupling constants are less than 2 Hz in all cases except for Thr-51. In the case of Thr-51 the observed value of $^3J_{HC^\alpha C^\beta H}$ differs by 2.3 Hz from that predicted on the basis of the crystal structure, but a change in χ_1 of less than 20° would be required to bring the values into exact agreement. Whether this discrepancy is due to experimental errors in the crystal structure, to a real difference between solution and crystal states, or to dynamical effects (see below) remains to be established. Even for this residue, however, there is no evidence that the predominant rotameric state in solution differs significantly from that in the crystal.

The initial conclusion from the above results is that the ϕ and χ_1 torsion angles describing the average structure in solution correspond to within 30° of the values defined from the crystal structure, and generally much better. It must be appreciated, however, that the coupling constants do not by themselves define unambiguously the torsion angles; for example, the g^- and t configurations (Figure 7) cannot be distinguished without specific distinction of the two H^β proton resonances, a difficult task even in simple peptides. Coincidental agreement of observed and calculated coupling constants at the level observed in this work cannot be ruled out but is considered to be unlikely. It is known, however, that the torsion angles in proteins are not fixed but undergo fluctuations, often on a very fast time scale (Karplus & McCammon, 1981, and references therein). Indeed for certain residues of lysozyme these fluctuations could be as large as ±60° (Delepierre et al., 1981; Olejniczak et al., 1981). These fluctuations will cause the coupling constants to be averaged over the range of torsion angles sampled by the protein. It can be seen from Figures 6 and 7 that, for the residues studied in the present work, the extent of this averaging must be limited. More detailed examination of data of the type presented here must permit the dynamical aspects of the structure to be investigated. For example, averaged coupling constants can be calculated from dynamical simulations (Karplus & McCammon, 1981), as demonstrated already for chemical shift values (Hoch et al., 1982) and used to test the predictions of these simulations. In addition, combination of coupling constant data with measurements of the time development of nuclear Overhauser effects (Delepierre et al., 1981; Olejniczak et al, 1981) may enable details of the dynamical behavior in solution to be defined empirically.

Overall, this study has demonstrated the feasibility of assigning main-chain proton resonances in a protein the size of lysozyme. For the assignment process to be successful, it was necessary to assume only a very general similarity between the average crystal and solution structures. The characteristics of these resonances may then be used to study in detail structural and dynamical features of the molecule, either by direct measurement or by comparison with crystallographic data, with other NMR measurements, with models for hydrogen exchange, and with predictions of dynamical simulations. At the present stage, the coupling constant measurements have demonstrated for the residues studied here a striking agreement between the torsion angles detected in the crystal structure and those relevant to the protein in solution.

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