confirmed as incorrect. Specificity toward β-FBP only, the keto form only, or both remains a viable explanation. We presently disfavor the sole use of the acyclic species because of (1) cyclic analogue binding evidence which will be presented in a subsequent paper and (2) the simple consideration that the K_M values of $\sim 10^{-6}$ M (Mehler, 1963; Rose & O'Connel, 1969) for total substrate would require an unreasonably low $K_M \simeq 10^{-8}$ M for the keto form.

These experiments rule out an anomerase activity for the liver enzyme in that neither α-FBP alone nor in conjunction with β-FBP is used as a substrate and no catalysis of muscle aldolase use of α-FBP by liver aldolase is seen as with yeast aldolase. Thus, in evolutionary terms, it would appear that the muscle enzyme may have been modified to form the liver enzyme which functions well in both directions as does the yeast. However, this was not done by employing the α form as a substrate or achieving an anomerase activity but simply by altering the active site so that the α anomer is not bound. Consequently, a rapid spontaneous anomerization proceeds to maintain α-FBP ⇄ β-FBP equilibrium. It remains to be seen whether this spontaneous rate is rapid enough or whether a separate anomerase activity is required. Interestingly, 2-keto-3-deoxygluconate-6-P aldolase has been shown to use the free carbonyl form of its substrate, thus allowing it to be coupled to the preceding enzyme gluconate-6-P dehydratase which produces an acyclic form of the substrate, the enol (Midelfort et al., 1977).

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A Structural Study of the Hydrophobic Box Region of Lysozyme in Solution Using Nuclear Overhauser Effects†

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ABSTRACT: Saturation of specific proton nuclear magnetic resonance (NMR) signals from residues in the hydrophobic box region of lysozyme (EC 3.2.1.17) has enabled negative nuclear Overhauser effects to be measured on the resonances of nearby protons. The assignments of resonances reported previously have been examined, and most have been confirmed. In conjunction with spin-decoupling methods, new assignments could be made so that assignments for some 70 resonances of 25 residues in lysozyme are now known. A high correlation was observed between the observed nuclear Overhauser effects and interproton distances calculated from crystallographic data. This indicates that the average structure of this region of lysozyme in the crystalline state is maintained in solution, that substantial populations of structures very different from this do not exist, and that the nuclear Overhauser technique can be applied in a straightforward manner to obtain structural data in solution at the 1-Å level.

A full understanding of the folding, conformation, and function of a protein requires that the structure and dynamics of individual groups of the molecule in solution are defined and that any changes in these caused, for example, by substrate binding or partial unfolding can be described. The information about protein structure in the crystalline state is essentially static and must be supplemented by information from other techniques. For this reason, ^{1}H NMR studies of an enzyme, lysozyme, in solution are being performed (McDonald & Phillips, 1970; Campbell et al., 1975a; Dobson, 1977).

Although the NMR studies of lysozyme have revealed novel information about the protein structure, dynamics, and conformational changes, the information obtained has not yet allowed local structure and dynamics in solution to be defined with great accuracy. The problem is that the large number of nuclei in a protein results in the NMR parameters for each nucleus being determined by the sum of a large number of

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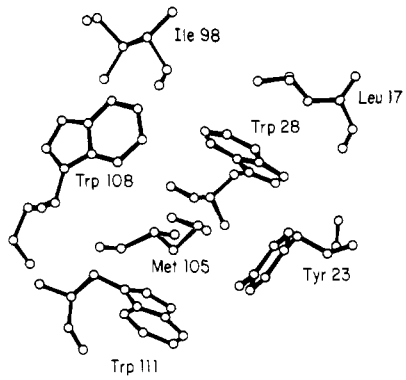

FIGURE 1: View of part of the hydrophobic box region of lysozyme drawn from the coordinates of Phillips. At least one resonance of each of the residues shown here was saturated in the present work.

different interactions with neighboring atoms. In order to obtain specific assignments and also structural and dynamic information, it is necessary to measure individual contributions to these summations. Earlier studies made use primarily of chemical methods to identify specific interactions between nuclei, but in this paper double-resonance methods are described. In particular, the nuclear Overhauser effect allows specific dipolar couplings between nuclei to be investigated (Noggle & Schirmer, 1971). The dipolar couplings are distance dependent and provide structural information relating to the separation of a saturated nucleus and nuclei whose resonances are observed to experience a change in intensity as a result of the saturation.

Large negative nuclear Overhauser effects were observed on specific resonances in the lysozyme spectrum following selective saturation of resonances of protons of the same residue (Campbell et al., 1974; Cassels et al., 1980). Smaller effects were detected between protons on different residues (Chapman et al., 1978), and this considerably enhances the applicability of the technique. Nuclear Overhauser effects have been observed in the proton NMR spectra of other proteins (Redfield & Gupta, 1971; Snyder et al., 1975; Campbell et al., 1976; Kalk & Berendsen, 1976; Gordon & Wüthrich, 1978; Wüthrich et al., 1978) and used to make assignments. The interpretation of the effects is not always straightforward, and in this work it has been possible to evaluate the nuclear Overhauser method for assignment and structural purposes by considering numerous Overhauser effects on resonances assigned by totally independent methods. The structural analysis is restricted here to the residues in the region of the lysozyme molecule called the hydrophobic box (Blake et al., 1965). This region is bordered by five aromatic residues, Tyr-20 and -23 and Trp-28, -108, and -111, and it contains other hydrophobic residues, notably Leu-17, Ile-98, and Met-105 (Figure 1). It borders on the active site of lysozyme, and conformational changes within it are established, particularly associated with the reorientation of Trp-108 (Blake et al., 1967; Perkins et al., 1977; Cassels et al., 1978). In the proton NMR spectrum, many resonances are well resolved, primarily because of substantial ring-current shifts from the aromatic residues (Sternlicht & Wilson, 1967; McDonald & Phillips, 1969). The experiments described in this paper confirm previous assignments and allow new ones to be made so that virtually every aromatic proton and methyl group resonance in this region has been located in the spectrum, along with other resonances.

Experimental Procedures

Lysozyme from hen egg white was obtained from Sigma Chemical Co. and dialyzed extensively at pH 3.0 before use. NMR samples were normally 7 mM in lysozyme and at pH 4.0 in D_2O. Where necessary, exchangeable hydrogens were replaced with deuterons by reversible thermal denaturation, and spectra were recorded normally at 57 °C.

^1H NMR spectra were recorded at 270 MHz with a Bruker spectrometer. Spin-decoupling difference, spin–echo double-resonance, and convolution difference techniques were all carried out as described previously (Campbell et al., 1973, 1975b; Campbell & Dobson, 1975). Nuclear Overhauser effects were measured as follows. A presaturation pulse of frequency f_2 and of varying length (0.1–4.0 s) was applied prior to accumulation of each free induction decay, which was followed by a variable delay (0–5.0 s) before the next presaturation pulse, as described previously (Campbell et al., 1977). After a fixed number of scans (typically 40), the free induction decay was stored, the presaturation pulse frequency changed to f_2', a short delay (~2 s) allowed for the frequency synthesizer to stabilize, and a second free induction decay collected for the same fixed number of scans and stored. Then, the frequency was changed back to f_2, the first free induction decay recalled into the computer memory, and the cycle repeated. In order to observe small effects, the two free induction decays were substracted, Fourier transformed, and phase corrected. Similar methods are described by Chapman et al. (1978) and Wagner & Wütrich (1979). The areas of the individual resonances in the difference spectra were measured relative to the area of resolved proton resonances in the individual spectra or, particularly in the case of saturation of solvent-exchangeable protons, relative to the area of the saturated resonance appearing in the difference spectrum. In order to observe spin decoupling of resonances appearing in the difference spectrum, time-shared irradiation at a frequency f_2'' was applied during the free induction decays.

A study of the time development of the Overhauser effect showed that the observed effects increased as expected as the length of the presaturation pulse increased. In view of these results, a value of the pulse length of 1.0 s was chosen for the assignment experiments described in this work. The delay between the end of the free induction decay and the next presaturation pulse was omitted, and the acquisition time was 0.5 s. With these methods, Overhauser effects of about 1% could be observed in about 1 h, a total of about 2000 accumulations. For more detailed study, longer accumulations of up to about 10 000 scans were made. In experiments where the Overhauser effects were studied under conditions far from the steady state, the length of the presaturation pulse was reduced to 0.25 s, and a delay of 1 s was allowed after the acquisition of the free induction decay and before the next pulse.

Calculations of distances between protons in the crystal structure (Blake et al., 1967) were carried out by using refined coordinates of the tetragonal crystal form provided by Professor D. C. Phillips (D. E. P. Grace and D. C. Phillips, unpublished data). Hydrogen atoms were generated in standard configurations, and it was assumed that for methyl groups the three hydrogen atoms were in a staggered configuration. For internuclear distances involving methyl groups or tyrosine ring protons, where rapid rotation results in averaging of the NMR signals from different protons, average distances are quoted in the text. In calculations relating to the Overhauser effects on methyl groups or tyrosine residues, an average distance weighted according to $1/r^6$ was employed. For Overhauser effects resulting from saturation of resonances from these groups, a summation of the effects calculated for each proton of the group was made.

Chemical shift values are quoted 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. Values for the spectrum at pH 5.3 and 57 °C are quoted in order to avoid complications with varying conditions.

Results

Assignment Studies. In order to study the residues involved in making up the hydrophobic box in lysozyme, it was necessary to saturate a number of different resonances. Those resonances which are well resolved were chosen in order to obtain selective and readily interpretable results and arose from different types of protons. The results are divided into sections according to the type of proton resonance saturated, but other experiments needed to interpret the observed Overhauser effects are included in each section. Full details of the time dependence of the effects will not be given here, but the Overhauser effects seen in the long-pulse experiment were shown to be qualitatively similar to the smaller effects seen in the short-pulse experiment. Thus, for the assignment studies, the long-pulse experiments will be illustrated to show the resonances more clearly. Assignments made previous to this work are included in Table I, along with references to the original papers. In the text of this paper, these references will, in general, therefore, not be given. Interproton distances are summarized in Table II, along with observed Overhauser effects.

(1) Tryptophan N(1) Protons. There are six tryptophan residues in lysozyme, and for each of these, the N(1)H and C(2)H resonances have been resolved and assigned by physical and chemical methods including chemical modifications (Cassels et al., 1978; Lenkinski et al., 1979). The protons of the six-membered ring of the indole moiety are not detectably spin–spin coupled to the N(1) or C(2) protons of the five-membered ring so that spin-decoupling methods cannot be used to relate the resonances of the six-membered ring to the assigned resonances of the five-membered ring. However, the N(1) proton should be strongly dipolar coupled to the C(2) proton and to the C(7) proton. Evidence for the N(1)H dipolar coupling to C(2)H has been obtained already by the observation of changes in transverse relaxation time (T_2) values of the C(2) proton resonances resulting from the exchange of N(1)H to N(1)D and from the observation of a nuclear Overhauser effect in one of the residues (Cassels et al., 1980).

Saturation of the resonances assigned to the N(1) proton of Trp-28, -108, -111, and -123 was carried out in turn as described under Experimental Procedures, and the observed negative nuclear Overhauser effects are shown in Figures 2 and 3. The experiments were carried out at lower temperatures than the other experiments described here in order that the NH exchange with solvent should be slow enough to permit sufficiently long accumulation times. Spectra of a number of different freshly dissolved samples were recorded and summed in order to improve the signal-to-noise ratios. Nevertheless, the exchange of the NH hydrogens of Trp-62 and -63 was too rapid to permit the experiment to be carried out for these residues. In each of the spectra, the most intense peak in the spectrum was in the aromatic region and corresponded to an Overhauser effect in the long-pulse experiment of 38 ± 4% at 37 °C (Table III). It arose in each case from the singlet resonance previously assigned to the C(2) proton of the residue to which the N(1)H resonance had been assigned. The next most intense peak in the difference spectrum was in each case an aromatic peak with an Overhauser effect of 21 ± 3%. These peaks could be observed in convolution difference spectra to be doublets and are assigned at once to the C(7) protons of the corresponding residues. The ratio of the Overhauser effects may be seen to be closely the same in

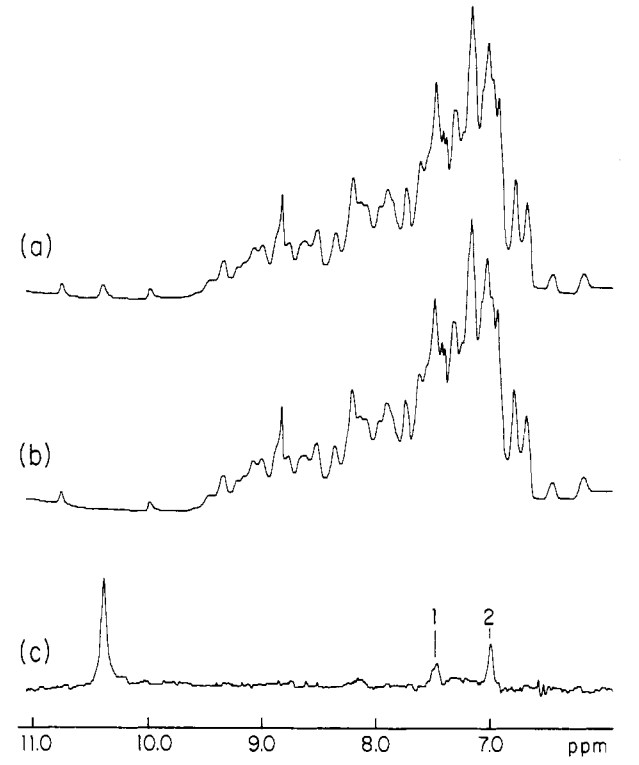

FIGURE 2: Spectra of 7 mM lysozyme at 37 °C recorded immediately after dissolution in D_2O at pH 4.0. In spectrum b a selective rf saturating field was applied at the position of the Trp-111 N(1)H proton resonance, while in spectrum a the field was applied at a frequency 100 Hz higher than this. The two spectra were recorded in the interleaved manner described under Experimental Procedures. Spectrum c is the difference between (a) and (b) expanded in the vertical scale by a factor of 8. The resonances labeled 1 and 2 arise from the C(7) and C(2) protons, respectively. In this and all difference spectra shown here, a Lorentzian line broadening of 2 Hz was applied.

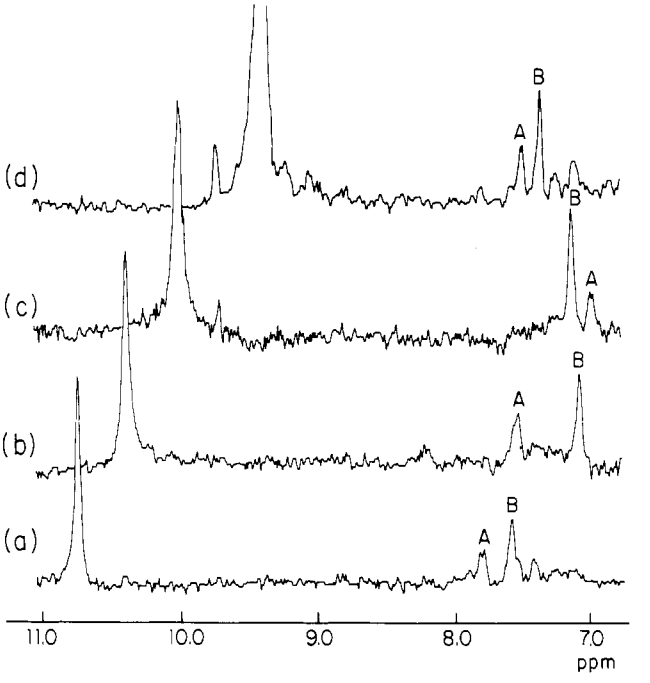

FIGURE 3: Difference spectra recorded as in Figure 2 but resulting from saturation of the N(1)H resonances of (a) Trp-123, (b) Trp-111, (c) Trp-108, and (d) Trp-28. Resonances labeled A and B are of C(7) and C(2) protons, respectively.

Table I: Assignments in the Lysozyme ^{1}H NMR Spectrum[a]

	C(α)	C(β)	C(γ_1)	C(γ_2)	C(δ_1)	C(δ_2)	C(ε)	N(1)	C(2)	C(3)	C(4)	C(5)	C(6)	C(7)
Lys-1	5.42[b]													
Leu-8			1.47[b]		−0.01[b]	0.53[b]								
Met-12							1.66[b]							
His-15						8.34[b,c]	7.23[b,c]							
Leu-17			0.68[b]		−0.10[b]	−0.63[b]								
Tyr-20									7.24[d]	6.98[d]		6.98[d]	7.24[d]	
Tyr-23									7.05[d]	6.71[d]		6.71[d]	7.05[d]	
Trp-28								9.36[e,f]	7.30[c]		6.76[h]	6.28[h]	6.82[h]	7.42[h]
Thr-51		3.76[b]	0.32[b]											
Tyr-53									7.09[d]	6.83[d]		6.83[d]	7.09[d]	
Leu-56			1.22[b]		0.28[b]	0.57[b]								
Trp-62								10.05[e,g]	7.03[e]					
Trp-63								10.21[e,g]	7.63[e]					
Ile-88					0.27[b]									
Val-92		1.92[b]	0.48[b]	0.60[b]										
Ala-95	3.93[h]	1.55[h]												
Ile-98	(2.88)[h]	1.56[b]	−2.10[f,h]	−0.26[b]	−0.01[b]									
			0.63[f,h]											
Val-99		2.43[h]	1.27[h]	1.20[h]										
Met-105	3.85[f]	−0.91[f]	(0.55)[h]				0.00[b]							
		0.44[f]												
Ala-107	3.87[b]	0.64[b]												
Trp-108								9.98[b,e,g]	7.08[b,e]		7.38[h]	6.49[h]	7.15[h]	6.92[h]
Val-109		2.19[b]	1.03[b]	1.14[b]										
Ala-110	4.31[b]	1.38[b]												
Trp-111								10.35[e,g]	7.03[e]					7.52[h]
Trp-123								10.68[e,g]	7.55[e]					7.75[h]

[a] Chemical shift values in ppm downfield of 4,4-dimethyl-4-silapentanesulfonate for 5 mM lysozyme in D_2O at pH 5.3 and 57 °C. The nomenclature denotes the carbon to which the proton is attached and is in agreement with the IUPAC-IUB Commission on Biochemical Nomenclature (1970) and with the published coordinates of lysozyme (Blake et al., 1967; Imoto et al., 1972). Underlining indicates that a nuclear Overhauser effect has been observed involving the proton concerned. References for the assignments are listed in footnotes *b–g*. Assignments are given or referenced only when the type of amino acid concerned has been identified by independent means, particularly observation of multiplet structure or spin decoupling. Exceptions are those given in parentheses and discussed in the text. [b] Campbell et al. (1975a). [c] Meadows et al. (1967). [d] Dobson et al. (1978). [e] Cassels et al. (1978). [f] Chapman et al. (1978). [g] Lenkinski et al. (1979). [h] This work.

each case and is 1.84 ± 0.2. In tryptophan itself, the ratio of the relaxation effects caused by the N(1) proton on the C(2) and C(7) protons was determined to be 1.86 from measurements of spin–lattice relaxation rates in H_2O and D_2O (Cassels et al., 1980). It should be noted that the ratio of $1/r^6$ for these protons, where r is the distance to the N(1) proton, is 1.97. The assignments may be made with confidence in light of these data.

For the doublet resonances of Trp-28 and Trp-108 observed in the difference spectra above, the positions of the spin–spin-coupled resonances have been identified by spin decoupling. For Trp-28, this doublet, at 7.42 ppm, is coupled to a triplet resonance at 6.82 ppm. This was confirmed by performing the Overhauser experiment at 57 °C, where the spin–spin coupling was resolved, and then repeating the experiment while irradiating during acquisition at 6.82 ppm. The decoupling caused by this irradiation was clearly visible. The triplet resonance at 6.82 ppm is also coupled to a clearly resolved triplet resonance at 6.28 ppm. The latter is also coupled to a doublet at 6.76 ppm. This permits an immediate assignment of all the aromatic proton resonances of Trp-28. In a previous paper, the coupling pattern centered on the triplet at 6.28 ppm was reported (Campbell et al., 1975b), and assignment to Trp-28 was proposed from chemical shift calculations (Perkins et al., 1977). One of the resonances was wrongly identified because of coincidental overlap of the triplet at 6.82 ppm with another triplet resonance, but the chemical shift calculations are in agreement with the present assignment.

A series of spin-decoupling experiments similar to those described above allowed identification of the resonances of Trp-108. The doublet resonance observed in the Overhauser difference spectra at 6.92 ppm is resolved in the normal spectrum and is decoupled by irradiation at 7.15 ppm. This irradiation also decoupled a resolved triplet at 6.49 ppm. Irradiation of this triplet decouples two resonances, and the decoupling difference spectra and spin–echo double-resonance spectra show that the resonance at 7.15 ppm is a triplet and that a doublet resonance at 7.38 ppm is also coupled to the irradiated triplet.

Saturation of the N(1)H resonances results in large Overhauser effects on the resonances of the adjacent protons of the aromatic rings. Smaller effects are also seen on other resonances. For example, saturation of the N(1) proton resonance of Trp-28 results in the appearance in the difference spectrum of a resonance of a methyl group assigned in previous work to Leu-17. By contrast, no effect is observed on any other methyl group resonance. An examination of the crystal structure of lysozyme shows that one of the two methyl groups of Leu-17 [the C(δ_2)H$_3$] is positioned close (4.73 Å) to N(1)H of Trp-28 (Figure 1) and that this is the nearest methyl group to this proton. In addition, no other tryptophan residue is close to Leu-17. Saturation of the N(1) proton resonance of Trp-111 results in no effect on the Leu-17 resonances but an observable effect on the resonance assigned to the methyl group of Met-105. In the crystal structure, Met-105 and Trp-111 are in close proximity (Figure 1). These experiments, there-

Table II: Nuclear Overhauser Effects and Interproton Distances[a]

residue	proton(s)	Met-12 C(ε)H$_3$	Leu-17 C(δ$_1$)H$_3$	Leu-17 C(δ$_2$)H$_3$	Tyr-20 C(2,6)H	Tyr-20 C(3,5)H	Tyr-23 C(2,6)H	Tyr-23 C(3,5)H	Trp-28 N(1)H	Trp-28 C(2)H	Trp-28 C(4)H	Trp-28 C(5)H	Trp-28 C(6)H	Trp-28 C(7)H	Leu-56 C(δ$_1$)H$_3$	Leu-56 C(δ$_2$)H$_3$	Trp-63 C(2)H	Val-92 C(γ$_1$)H$_3$	Val-92 C(γ$_2$)H$_3$	Ala-95 C(α)H	Ala-95 C(β)H$_3$
Leu-17	C(δ$_2$)H$_3$	0.9 (7.16)	5.0 (3.36)		0.6 (4.82)	1.2 (6.28)			* (4.73)	* (5.62)	* (5.22)	* (5.17)	1.6 (4.69)	* (4.19)				2.4 (4.44)	0.7 (5.82)		
Tyr-23	C(3,5)H						*b* (3.72)														
Trp-28	N(1)H			* (4.73)						+ (2.36)				+ (2.81)							
Trp-28	C(5)H	* (6.20)	* (5.13)	* (5.17)							10.3 (2.47)		12.4 (2.51)	* (4.32)	* (4.66)	* (3.91)				* (4.34)	2.7 (3.27)
Ile-98	C(γ$_1$)H(2)																* (5.24)			* (4.00)	
Ile-98	C(γ$_2$)H$_3$																* (6.18)				
Met-105	C(β)H(1)						* (4.73)	1.9 (3.70)													
Trp-108	C(5)H											* (3.50)	* (3.01)								* (5.60)

residue	proton(s)	Ile-98 C(α)H	Ile-98 C(β)H	Ile-98 C(γ$_1$)-H(1)	Ile-98 C(γ$_1$)-H(2)	Ile-98 C(γ$_2$)-H$_3$	Ile-98 C(δ)-H$_3$	Val-99 C(γ$_1$)-H$_3$	Val-99 C(γ$_2$)-H$_3$	Met-105 C(α)H	Met-105 C(β)-H(2)	Met-105 C(γ)-H(1)	Met-105 C(γ)-H(2)	Met-105 C(ε)H$_3$	Ala-107 C(β)H$_3$	Trp-108 C(2)H	Trp-108 C(4)H	Trp-108 C(5)H	Trp-108 C(6)H	Trp-108 C(7)H	Trp-111 C(2)H	Trp-111 C(7)H
Trp-28	C(5)H							* (5.62)	* (5.31)					* (4.46)				* (3.50)	4.7 (2.94)	* (3.84)		
Ile-98	C(γ$_1$)H(2)	8.2 (2.64)	4.3 (2.60)	20.3 (1.76)		2.2 (3.90)	3.9 (2.67)												+ (5.10)	* (5.31)		
Ile-98	C(γ$_2$)H$_3$	8.4 (3.30)	10.2 (2.65)	8.1 (2.97)	* (3.90)		+ (3.91)								5.6 (3.33)			* (4.23)	+ (3.83)	* (4.51)		
Met-105	C(β)H(1)									6.5 (3.04)	15.9 (1.76)		+ (2.32)	1.7 (4.54)								2.6 (3.85)
Trp-108	N(1)H															b (2.35)				b (2.77)		
Trp-108	C(5)H					0.6 (4.23)		0.5 (4.86)	1.1 (3.26)	4.0 (2.69)		5.5 (2.70)		0.7 (4.69)	* (4.87)		11.4 (2.47)		10.0 (2.50)			
Trp-111	N(1)H													b (4.71)							b (2.38)	b (2.75)

[a] Measured at 57 °C using 7 mM lysozyme in D_2O at pH 4.0. Values of Overhauser effects are negative and are given in percent for the short (0.25 s)-pulse experiment described under Experimental Procedures. The columns indicate the saturated resonances and the rows the observed resonances. The numbers in parentheses are the internuclear distances (in angstroms) from the refined crystallographic coordinates calculated as described under Experimental Procedures. For methyl groups and tyrosine ring protons, a simple average distance is given. The symbol * indicates that the effect is too small to measure in the short-pulse experiment but can be observed in the long-pulse experiment. The symbol + indicates that the peak is observed but is not well enough resolved to allow a significant number to be obtained. The letter b indicates that the effect was seen in the long-pulse experiment but was not carried out with the short pulse.

Table III: Nuclear Overhauser Effects from Saturation of Tryptophan N(1) Proton Resonances[a]

residue	Overhauser effect (%) C(2)H	Overhauser effect (%) C(7)H	ratio of effect C(2)H/C(7)H
28[b]			1.83
108	37.1	18.5	2.01
111	42.0	25.1	1.67
123	34.6	18.8	1.84

[a] Measured at 37 °C with the 1.0-s pulse as described under Experimental Procedures. The effects are all negative. [b] Absolute values of the Overhauser effect could not be obtained because the extent of exchange of the Trp-28 N(1)H resonance in a given spectrum could not be measured accurately because of resonance overlap.

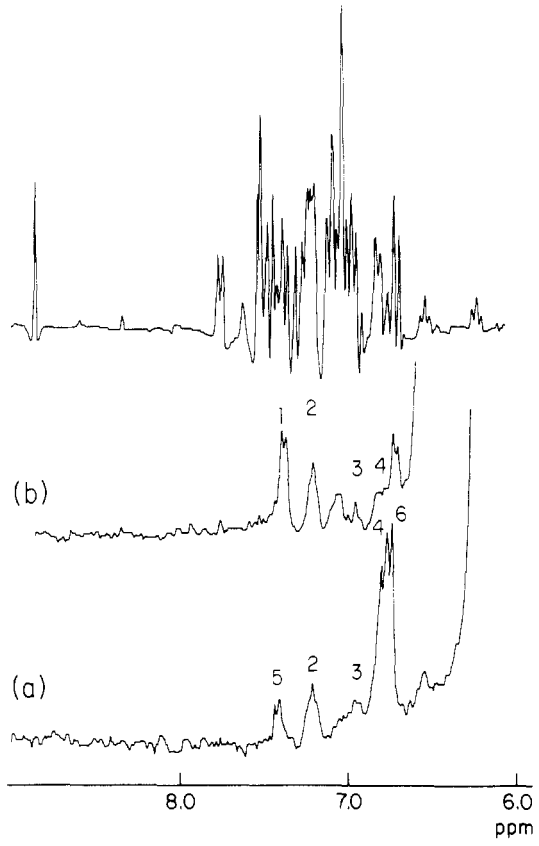

FIGURE 4: Nuclear Overhauser difference spectra in the aromatic proton region resulting from saturation of the C(5) proton resonances of (a) Trp-28 and (b) Trp-108. Above this and subsequent figures is a convolution difference spectrum under the same conditions. Resonances 1, 2, and 3 are of the C(4), C(6), and C(7) protons of Trp-108, and 4, 5, and 6 are of the C(6), C(7), and C(4) protons of Trp-28.

fore, totally confirm the assignments made previously and would be totally inconsistent with any alternative assignment. Further confirmation comes from the observation of nuclear Overhauser effects from the $C(\delta_2)H_3$ methyl group of Leu-17 on all the Trp-28 aromatic proton resonances and from the $C(\beta)H_2$ group of Met-105 on Trp-111 aromatic proton resonances. These will be described below.

(*2*) *Aromatic CH Protons.* The C(5) proton resonances of both Trp-28 and Trp-108 are clearly resolved in the spectrum. Saturation of the Trp-28 C(5) proton resonance results in several well-resolved peaks in the difference spectrum (Figures 4 and 5). In the aromatic proton region, the resonances of other Trp-28 protons, C(4)H, C(6)H, and C(7)H, are prominent (Figure 4). These protons are all within 4.5 Å of the C(5) proton, and the C(4) and C(6) protons are within 2.6

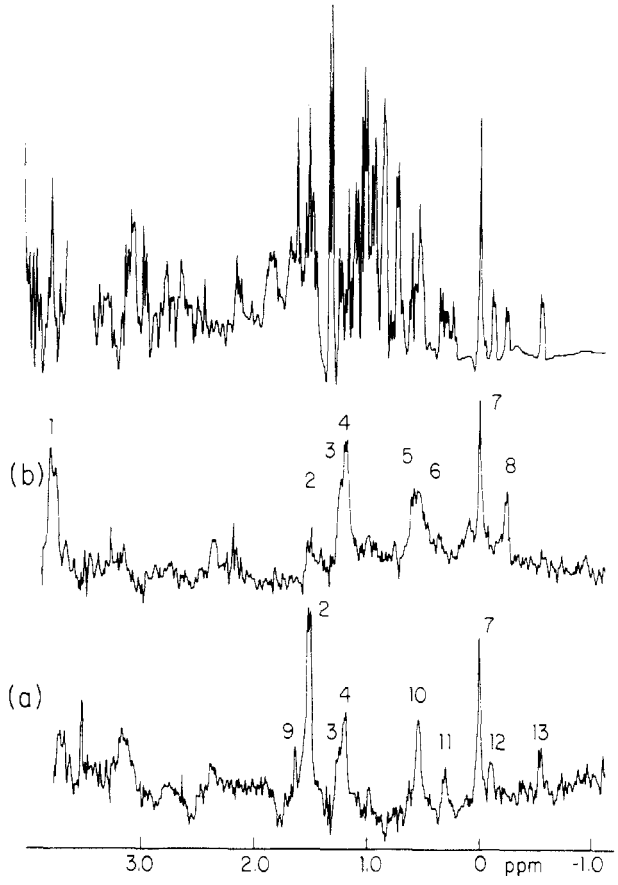

FIGURE 5: Nuclear Overhauser difference spectra in the methyl group and methylene proton region resulting from saturation of the C(5) proton resonances of (a) Trp-28 and (b) Trp-108. Assignments are (1) Met-105 $C(\alpha)H$, (2) Ala-95 $C(\beta)H_3$, (3) Val-99 $C(\gamma_2)H_3$, (4) Val-99 $C(\gamma_1)H_3$, (5) Ala-107 $C(\beta)H_3$, (6) Met-105 $C(\gamma)H$, (7) Met-105 $C(\epsilon)H_3$, (8) Ile-98 $C(\gamma_2)H_3$, (9) Met-12 $C(\epsilon)H_3$, (10) Leu-56 $C(\delta_2)H_3$, (11) Leu-56 $C(\delta_1)H_3$, (12) Leu-17 $C(\delta_1)H_3$, and (13) Leu-17 $C(\delta_2)H_3$.

Å. Another large peak is seen in this region of the difference spectrum and is that assigned in the previous section to the C(6) proton of Trp-108. In the crystal structure, the distance between the C(5) proton of Trp-28 and the C(6) proton of Trp-108 is only 2.94 Å; see Figure 1. Smaller peaks corresponding to the resonances of the C(5) and C(7) protons of Trp-108 can be seen; these are within 4 Å of the Trp-28 C(5) proton in the crystal structure.

In the methyl group region of the difference spectrum, a number of well-resolved peaks can be identified (Figure 5). The largest peak has not been assigned previously, but other prominent peaks have been assigned to the methyl groups of Leu-17, Leu-56, and Met-105. In the crystal structure, the methyl groups of these three residues are all within 5.2 Å of the C(5) proton (Table II). The methyl group of Met-12 is 6.2 Å from the C(5) proton, but a very small effect on the resonance of this group can be seen in the spectrum (Figure 5). In the crystal structure, the closest methyl group to the C(5) proton of Trp-28 is that of Ala-95 at 3.27 Å, whose resonance has not been identified previously. Also within 6 Å are both methyl groups of Val-99 and again resonances of this residue have not been identified previously. We shall return to these resonances later.

Saturation of the C(5) proton resonance of Trp-108 results in large observed effects on the resonances of the adjacent C(4) and C(6) protons and in a smaller effect on the resonance of the C(7) proton. These observations are entirely analogous to those for Trp-28 (Figure 4). Other peaks in the aromatic

proton region of the difference spectrum arise from the C(5) and C(6) protons of Trp-28, both of which are within 4 Å of the C(5) proton of Trp-108.

In the methyl group region of the spectrum (Figure 5), resonances assigned previously to the methyl groups of Ile-98 and Met-105 can be identified in the difference spectrum. A peak assigned to the methyl group of either Ala-107 or Ala-31 is also observed, as are peaks corresponding to those seen in the difference spectrum following saturation of the Trp-28 C(5) proton resonance. There are also two prominent CH resonances, at 0.55 and 3.85 ppm. The latter has been assigned to C(α)H of Met-105 (see Aliphatic CH Protons), and the former is assigned to a C(γ)H_2 proton of Met-105 as described under Aliphatic CH Protons. In the crystal structure, the methyl groups of Ile-98 and Met-105 are close to the C(5) proton of Trp-108 (4.23 and 4.69 Å, respectively). C(α)H and one of the C(γ)H_2 protons of Met-105 are also very close (2.69 Å and 2.70 Å, respectively).

For both the experiments described in this section, the total consistency between the observed nuclear Overhauser effects on assigned resonances and the proximity of protons in the crystal structure prompts assignment of the prominent but previously unassigned resonances observed in the difference spectra. In order that the assignments are rigorous, the identification of the type of residue from which the observed resonance arises was investigated by spin decoupling. First, consider the peak at 0.64 ppm, which has been assigned in earlier work to arise from an alanine or threonine residue and then to either Ala-31 or Ala-107. This is seen in the Overhauser difference spectrum following saturation of the Trp-108 C(5) proton resonance. In the crystal structure, Ala-107 is 4.87 Å from this proton while Ala-31 is 6.92 Å away. Assignment to Ala-107 may therefore be made, and this is further confirmed in the next section where an Overhauser effect on the same resonance is observed when the Ile-98 C(γ_2)H_3 group resonance is saturated. Next, consider the resonances at 1.2 ppm, which are seen in the difference spectra following irradiation of the C(5) proton resonances of both Trp-28 and -108. These resonances are both decoupled by irradiation at 2.43 ppm, permitting their assignment to the two methyl groups of a valine or leucine residue. Examination of the crystal structure permits immediate assignment to Val-99, whose methyl groups are within 6 Å of the C(5) protons of the two residues. Next, the resonance at 1.55 ppm, which is seen clearly following saturation of the C(5) proton of Trp-28, is just resolvable in the difference spectrum following irradiation of the C(5) proton of Trp-108. This resonance is decoupled by irradiation at 3.93 ppm, permitting assignment to be made to a methyl group of alanine or threonine. This can then be assigned to the methyl group of Ala-95, which in the crystal structure is 3.27 Å from the C(5) proton of Trp-28 and 5.60 Å from that of Trp-108.

Finally, in this section, saturation of the resolved C(3,5) proton resonance of Tyr-23 at 6.71 ppm is considered. This results in the expected large Overhauser effect on the C(2,6) proton resonance at 7.05 ppm. An additional prominent peak occurs in the Overhauser difference spectrum, and this is the peak of the C(7) proton of Trp-111. In the crystal structure, this proton is 2.76 Å from one of the C(3,5) protons of Tyr-23. No significant resonances could be resolved in the methyl group region, in keeping with the crystal structure, which shows no methyl group within 6 Å of the Tyr-23 C(3,5) protons. Already, the Overhauser effects are forming a map of this region of the protein (Figure 10) which will be further extended in the next sections.

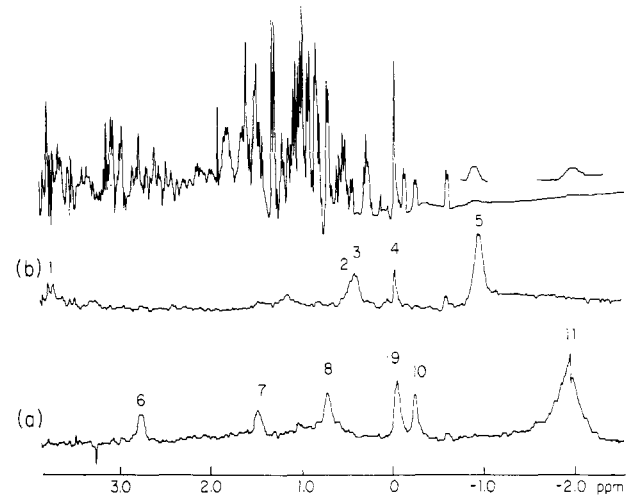

FIGURE 6: Nuclear Overhauser difference spectra in the methyl group and methylene proton region resulting from saturation of (a) the C(γ)H_2 resonance of Ile-98 at −2.10 ppm and (b) the C(β)H_2 resonance of Met-105 at −0.91 ppm. Resonances 1–5 are of the C(α)H, C(γ)H(1), C(β)H(2), C(ϵ)H_3, and C(β)H(1) protons of Met-105. Resonances 6–11 are of the C(α)H, C(β)H, C(γ_1)H(1), C(δ)H_3, C(γ_2)H_3, and C(γ_1)H(2) protons of Ile-98.

(*3*) *Aliphatic CH Protons.* There are two resonances in the high-field region of the lysozyme spectrum which are very well resolved. First, the resonance at −2.10 ppm is considered. Spin decoupling of the resonance assigned to the C(δ)H_3 group of Ile-98 occurs as a result of irradiation at this position, showing that the peak arises from one of the protons of the C(γ_1)H_2 group of Ile-98. Saturation of the resonance gives rise to many peaks in the difference spectrum, particularly in the high-field region (Chapman et al., 1978). The most prominent peak is at 0.63 ppm (Figure 6). This peak is of the other C(γ_1)H_2 proton of Ile-98, as spin decoupling of the C(γ_2)H_3 group occurs from irradiation at this position. The two methyl group resonances of Ile-98 are seen clearly, as is the resonance of the C(β)H proton at 1.56 ppm. The latter has been assigned by observation of spin decoupling of the C(γ_2)H_3 resonance which results from irradiation at 1.56 ppm. A resonance at 2.88 ppm is tentatively assigned to the C(α) proton of Ile-98, but spin decoupling has not yet been conclusively demonstrated. All the protons of Ile-98 are within 5 Å of both of the C(γ_1) protons of this residue in the crystal structure. A prominent resonance at 3.93 ppm in the difference spectra is in the position of the C(α) proton resonance of Ala-95, which is also very close (4.0 Å) to the C(γ_1)H(2) proton of Ile-98.

In the aromatic proton region, there are several resonances which are not well resolved (Figure 7). Of the resolved resonances, those of the Trp-108 C(7) proton and the Trp-63 C(2) proton have been previously assigned. Both of these protons are close to the Ile-98 C(γ_1)H_2 protons in the crystal structure, as are other protons of Trp-63. The relative magnitudes of the effects on the different protons (see below) show that assignment to the C(γ_1)H(2) proton is more likely than to the C(γ_1)H(1) proton. However, this depends critically on the accuracy of the crystallographic coordinates.

Saturation of the resonance at −0.91 ppm gives quite different peaks in the difference spectra from those discussed above (Figures 6 and 7). The resonance is assigned to be from a C(β)H_2 group because it is spin coupled to a resonance at 3.85 ppm (Chapman et al., 1978) whose chemical shift indicates that it arises from a C(α) proton. Saturation of the resonance at −0.91 ppm gives a large effect on a resonance at 0.44 ppm assigned to the other C(β) proton. Large effects

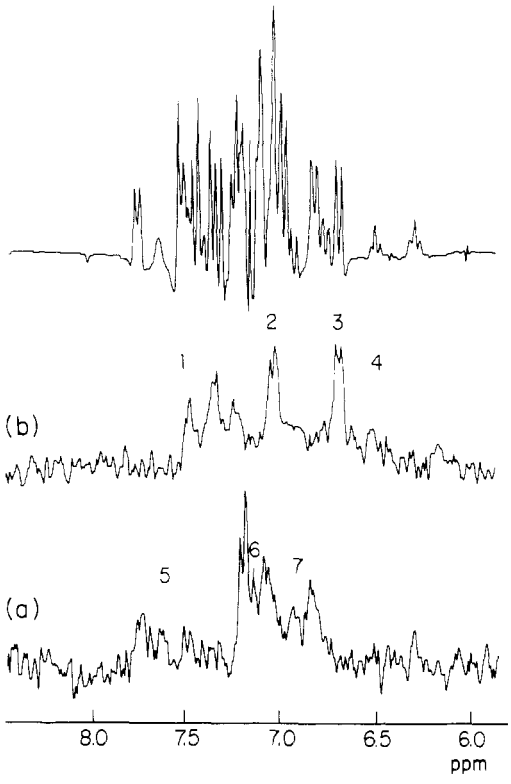

FIGURE 7: Nuclear Overhauser difference spectra in the aromatic proton region resulting from saturation of (a) the $C(\gamma_1)H_2$ resonance of Ile-98 at −2.10 ppm and (b) the $C(\beta)H_2$ resonance of Met-105 at −0.91 ppm. The assignments which are known are (1) Trp-111 C(7)H, (2) Tyr-23 C(2,6)H, (3) Tyr-23 C(3,5)H, (4) Trp-108 C(5)H, (5) Trp-63 C(2)H, (6) Trp-108 C(6)H, and (7) Trp-108 C(7)H. The additional resonances in (a) arise primarily from Trp-63 protons and in (b) from Trp-111 protons (see text).

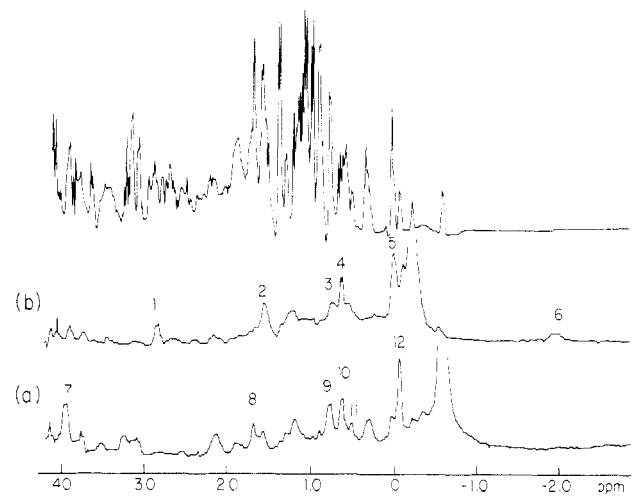

FIGURE 8: Nuclear Overhauser difference spectra in the methyl group and methylene proton region resulting from saturation of (a) the $C(\delta_2)H_3$ resonance of Leu-17 at −0.63 ppm and (b) the $C(\gamma_2)H_3$ resonance of Ile-98 at −0.26 ppm. Assignments are (1) Ile-98 C(α)H, (2) Ile-98 C(β)H, (3) Ile-98 $C(\gamma_1)H(1)$, (4) Ala-107 $C(\beta)H_3$, (5) Ile-98 $C(\delta)H_3$, (6) Ile-98 $C(\gamma_1)H(2)$ (7) Leu-17 C(α)H, (8) Met-12 $C(\epsilon)H_3$, (9) Leu-17 C(γ)H, (10) Val-92 $C(\gamma_2)H_3$, (11) Val-92 $C(\gamma_1)H_3$, and (12) Leu-17 $C(\delta_1)H_3$.

are visible on the methyl group resonance of Met-105 (Figure 6) and in the aromatic region (Figure 7) on resonances assigned previously to the Tyr-23 C(3,5) and C(2,6) protons, on the C(5) proton resonance of Trp-108, and on the C(7) proton resonance of Trp-111. The resonance at −0.91 ppm can be assigned to one of the $C(\beta)H_2$ protons of Met-105, the only assignment consistent with the crystal structure interproton distances and also in accord with previous evidence including ring-current calculations (Cowburn et al., 1970; Chapman et al., 1978). From a consideration of the crystal structure, and the high intensity in the Overhauser difference spectrum of the Tyr-23 resonance, the assignment to the $C(\beta)H(1)$ proton is more likely than to the $C(\beta)H(2)$ proton, an assignment in accord with ring-current shift calculations (Cowburn et al., 1970). Protons other than those described above are within a few angstroms of the $C(\beta)H_2$ protons of Met-105. The shoulder on the peak at 0.44 ppm probably arises from a $C(\gamma)H_2$ proton and is in exactly the position observed for one of the peaks in the difference spectrum resulting from saturation of the C(5) proton of Trp-108 (Figure 5). In the aromatic proton region, peaks between 7.2 and 7.5 ppm are likely to arise from Trp-111 (Figure 1) but are too close together for spin decoupling to be carried out. The distances to the relevant protons are given in Table II.

(*4*) *Methyl Groups.* Two methyl groups from residues in the hydrophobic box region of lysozyme give rise to very well resolved resonances. One of these, at −0.63 ppm, has been assigned previously to Leu-17 and is assigned here to the $C(\delta_2)H_3$ group because of the Overhauser effect from the N(1)H proton of Trp-28. This is also in accord with ring-current calculations (Cowburn et al., 1970). The other methyl group is at −0.26 ppm and has been assigned to the $C(\gamma_2)H_3$ group of Ile-98. Nuclear Overhauser effects on these resonances have been seen from saturation of other resonances, as described in the previous sections.

Saturation of the $C(\gamma_2)H_3$ group resonance of Ile-98 results in many peaks in the difference spectrum (Chapman et al., 1978). Comparison of Figures 6 and 8 and Figures 7 and 9 shows that many of these peaks were also seen following saturation of the $C(\gamma_1)H(2)$ proton of the same residue. The two prominent methyl group resonances (Figure 8) are of the $C(\delta)H_3$ group of Ile-98 and of the Ala-107 methyl gruop, which is 3.33 Å distant in the crystal structure. Prominent aliphatic CH proton resonances are those which have been assigned to the $C(\gamma_1)H_2$ protons and the C(α)H proton of Ile-98. In the aromatic proton region (Figure 9), resonances of several Trp-108 protons are seen, those of the C(5), C(6), and C(7) protons, which are within 5 Å of the Ile-98 $C(\gamma_2)H_3$ group in the crystal structure. The C(2) proton resonance of Trp-63 is also well resolved, being 6.18 Å distant in the crystal structure. Other prominent resonances are from even closer protons of Trp-63, which have not yet been fully assigned.

Saturation of the $C(\delta_2)H_3$ group resonance of Leu-17 gives rise to peaks in the difference spectra from many assigned resonances. Of the methyl group resonances, those of the $C(\delta_1)H_3$ group of Leu-17, of the two methyl groups assigned to Val-92 (at 0.48 and 0.60 ppm), and of the Met-12 methyl group (at 1.66 ppm) are readily identified. These are all close to the saturated protons. Numerous other resonances of CH protons are resolved in the difference spectrum (Figure 8) but have not yet been rigorously assigned. The aromatic proton region of the difference spectrum is of considerable interest. That shown in Figure 9 has been obtained for a sample in which NH protons have only partially been exchanged for deuterons. Every aromatic proton resonance of Trp-28, including those of the N(1) proton (Chapman et al., 1978) and the C(2) proton, can be identified in the spectrum. Reference to Figure 1 explains this observation, for in the crystal structure the $C(\delta_2)H_3$ group of Leu-17 is positioned directly over the Trp-28 ring, and all these protons are within 6 Å of the methyl group. Also observed in the difference spectrum are the two resonances assigned previously to Tyr-20. The nearest C(2,6)

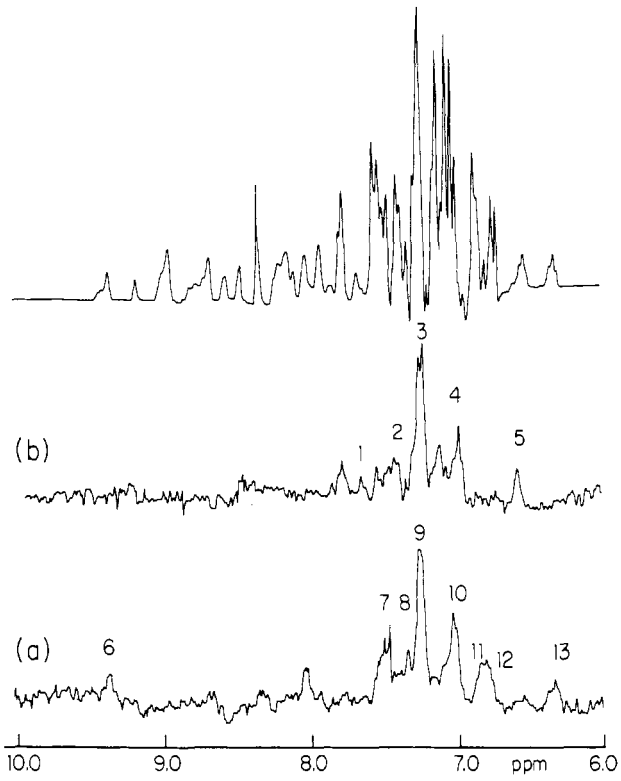

FIGURE 9: Nuclear Overhauser difference spectra in the aromatic proton region resulting from saturation of (a) the $C(\delta_2)H_3$ resonance of Leu-17 at −0.63 ppm and (b) the $C(\gamma_2)H_3$ resonance of Ile-98 at −0.26 ppm. Note the similarity of spectrum 7a to spectrum 9b. Assignments are (1) Trp-63 C(2)H, (2) Trp-108 C(7)H, (3) Trp-108 C(6)H, (4) Trp-108 C(7)H, (5) Trp-108 C(5)H, (6) Trp-28 N(1)H, (7) Trp-28 C(7)H, (8) Trp-28 C(2)H, (9) Tyr-20 C(2,6)H, (10) Tyr-20 C(3,5)H, (11) Trp-28 C(6)H, (12) Trp-28 C(4)H, and (13) Trp-28 C(5)H.

proton of Tyr-20 is 4.23 Å from the $C(\delta_2)H_3$ group of Leu-17 although the nearest C(3,5) proton is at 5.83 Å. Also in the low-field region of the difference spectrum of Figure 9 is a resonance of a peptide NH proton. In separate experiments on samples in which NH hydrogens have not been exchanged with deuterium, we have observed numerous Overhauser effects to and from the NH protons. Assignment of these resonances will be described elsewhere.

Structural Studies. Overhauser effect measurements in protein NMR have much greater significance than merely as assignment aids. The schematic diagram shown in Figure 10 summarizes the Overhauser effects observed in the lysozyme hydrophobic box. This diagram in itself is an outline structure of this region of the protein in solution. The large number of measured nuclear Overhauser effects between assigned protons in the hydrophobic box region of lysozyme gives rise to the opportunity of examining the dependence of the magnitude of the Overhauser effect on interproton distance and, hence, to study the solution structure in detail.

The long-pulse experiments described here approach the steady-state condition for the homonuclear Overhauser effect (η_{ik})

$$\eta_{ik} = \frac{\sigma_{ik}}{\rho_i} - \sum_{i \neq j,k} \frac{\sigma_{ij}\eta_{jk}}{\rho_i} \qquad (1)$$

where i is the observed spin, k the saturated spin, and j is any other coupled spin. ρ_i and σ_{ij} are the dipolar direct and cross relaxation rates, respectively (Solomon, 1955; Kalk & Berendsen, 1976), and depend on the correlation times for molecular motion and the distances between nuclei. For isotropic tumbling

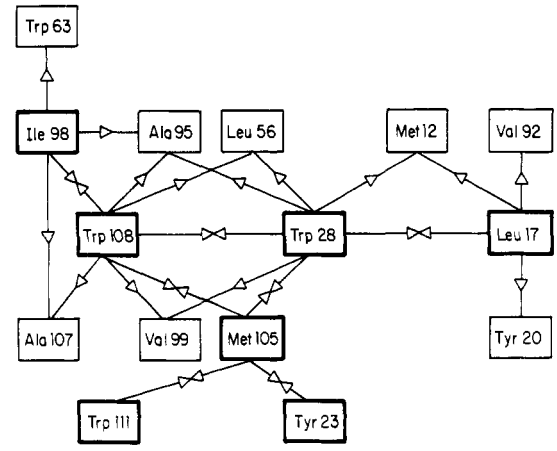

FIGURE 10: Schematic structure of the hydrophobic box region of lysozyme showing the Overhauser effects described in this paper. The residue to which an arrow points indicates that an Overhauser effect was seen on this residue from the residue from which the arrow originates. Residues whose resonances have been saturated are indicated by a heavy border.

$$\rho_i = \frac{\hbar^2\gamma^4}{10} \sum_{i \neq j} \frac{1}{r_{ij}^6} \left[\tau_c + \frac{3\tau_c}{1 + \omega^2\tau_c^2} + \frac{6\tau_c}{1 + 4\omega^2\tau_c^2} \right] \qquad (2)$$

and

$$\sigma_{ij} = \frac{\hbar^2\gamma^4}{10} \frac{1}{r_{ij}^6} \left[\frac{6\tau_c}{1 + 4\omega^2\tau_c^2} - \tau_c \right] \qquad (3)$$

where r_{ij} is the distance apart of spins i and j, γ is the magnetogyric ratio of the proton, ω is the frequency of measurement, and τ_c is the correlation time for time-dependent isotropic molecular motion.

For saturation of spin k, therefore, the observed nuclear Overhauser effect on a spin i depends on a complex summation of relaxation rates linking spins i and k and their neighbors. For large molecules, where $\omega\tau_c \gg 1$, σ_{ik} and σ_{ij} become large and negative. This can result in efficient diffusion of magnetization among coupled spins (Hull & Sykes, 1975; Kalk & Berendsen, 1976; Bothner-By & Johner, 1978). The opposite signs of σ and ρ (eq 2 and 3) mean that the maximum value of η_{ik} is −1, and in the limiting case of spin diffusion, total saturation of the spectrum can occur from the saturation of one spin. The data presented in Figures 2–9 and described above show that this limiting case is not approached experimentally in this work, although $\omega\tau_c$ is nearly 10 under these conditions. Indeed, the effects are much less than 1% for distances greater than 8 Å between the saturated and observed spins, although for short distances, intensity changes of up to 50% were observed. It is apparent from the complexity of eq 1, however, that interproton distances cannot be calculated from experimental observations in a simple manner. The problem becomes more straightforward if the length of the saturation pulse is reduced (Wagner & Wüthrich, 1979). In the limit of a short pulse of length t

$$\eta_{ik} = \sigma_{ik}t \qquad (4)$$

Provided that the correlation time τ_c is the same for the different nuclei, the relative effect of irradiation of spin k in different nuclei i and j is simply

$$\eta_{ik}/\eta_{jk} = (r_{jk}/r_{ik})^6 \qquad (5)$$

Because the magnitudes of the Overhauser effects are reduced as pulse lengths are reduced, acceptable signal-to-noise

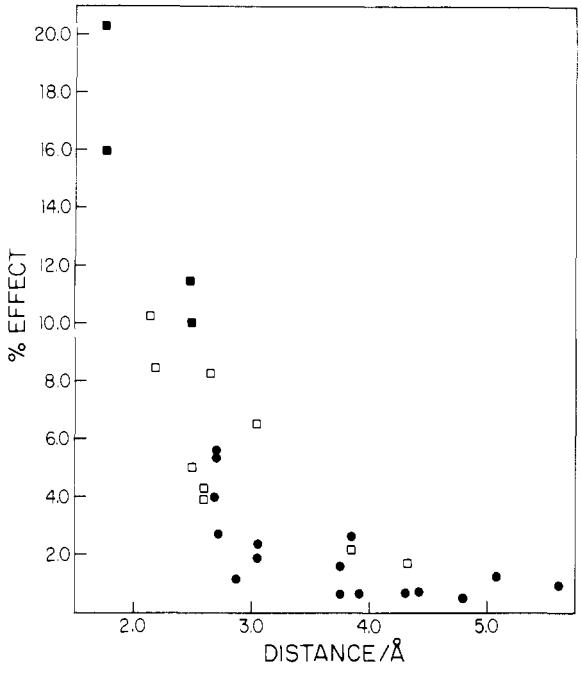

FIGURE 11: Plot of observed Overhauser effect in the short-pulse experiment against interproton distance (calculated as described under Experimental Procedures). Closed squares indicate that the interproton distance is fixed by the nature of the group (e.g., in a CH_2 group). Open squares indicate that both protons are of the same residue and closed circles that they are of different residues.

ratios could not be obtained for pulses shorter than 0.25 s. A pulse repetition time of 1.76 s was found to be sufficient for the system to relax between pulses while still being short enough to obtain acceptable spectra in reasonable lengths of time. With these conditions, the Overhauser experiments described above for assignment purposes were repeated. The areas of the peaks in the difference spectra were measured, and the resulting values of Overhauser effects are given in Table II, along with interproton distances calculated from the X-ray coordinates. It was found that no effects were measurable for interproton distances of more than 5.5 Å, and all effects for protons further apart than 3.5 Å were much less than 5%. However, interproton distances of less than 2.0 Å (i.e., between protons of a methylene group) were characterized by effects of more than 15%. It was therefore clear that the effect of shortening the pulse was to reduce considerably the Overhauser effects on more distant protons while maintaining fairly large effects on nearby protons.

In Figure 11, the observed Overhauser effects are plotted against the calculated interproton distances. The clear correlation at once confirms the qualitative analysis made in the previous section for the long-pulse experiments. There is a simple relationship between relative observed effects and calculated distances which is to the level of ±1 Å and which can be standardized by observation of effects between conformationally rigid protons. The prediction from eq 4 is that the relative Overhauser effects should be inversely proportional to the sixth power of the internuclear distances. While our present data are neither sufficiently extensive nor accurate for a critical test, the depedence is close to this, although somewhat larger effects than expected are seen for longer distances (Dobson, et al., 1980). This arises, at least in part, because the pulse length of 0.25 s is not short enough for the simple approximation to be totally valid. Nevertheless, the data indicate quite clearly that experimental estimates of some 30 interproton distances in the hydrophobic box region of lysozyme all correlate to better than ±1 Å with distances measured from crystallographic coordinates.

Discussion

The experimentally observed nuclear Overhauser effects are highly specific under the conditions used in this work. There is a simple qualitative correlation of observed Overhauser effects with the positions of residues determined for the crystal structure even when measured under conditions close to the steady state. This correlation has allowed all assignments made previously by independent methods to be confirmed and has permitted the firm assignment of new resonances. In all, some 70 resonances of 25 residues have now been assigned for lysozyme, and these are summarized in Table I.

In order to obtain structural information, the pulse lengths in the Overhauser experiments were reduced, sacrificing the signal-to-noise ratio for selectivity. The high correlation of the magnitude of the Overhauser effects observed under these conditions with crystallographic interproton distances has considerable significance for the applicability of this method in assignment and particularly in structure analysis. In order to estimate interproton distances to about ±1 Å from experiments of the type described here, it is merely necessary to standardize observed effects with those between conformationally rigid protons (Figure 11).

The results for lysozyme show that the average positions of groups in the hydrophobic box relative to one another are closely similar in solution to those determined in the crystal. The degree of similarity involves at the lowest level the relative positions of different residues in the protein (Figure 10). Then, at the next level, it requires that details of the structure, such as the relative orientation of the indole rings of Trp-28 and -108 and the conformation of side chains like Ile-98, are maintained in solution. This result is to be compared with the high level of agreement between coordinates derived from different crystal forms (Moult et al., 1976) and with low values of temperature factors derived from the crystallographic analysis (Blake et al., 1978; Sternberg et al., 1979) for this region of lysozyme. Previously, however, NMR studies have shown clearly that a variety of dynamical processes exist. Rotational motions of aromatic rings were specifically defined, and other motions were outlined in general terms (Campbell et al., 1975c; Dobson 1977). The data reported in this paper do not, of course, suggest that dynamical processes do not occur. The Overhauser effects are averaged over the various conformational states in the molecule and reflect the existence and populations of these states. The high degree of correlation between the measured Overhauser effects and the crystal structure in this region of lysozyme does indicate, however, that significant populations of conformational states very different from this average do not exist.

The data presented in this paper are only an initial sampling of the information available from these methods. More detailed measurements under a variety of conditions will permit further assignments to be made, and detailed analysis should permit average and dynamical aspects of the solution conformation to be defined more closely both for this region of the protein and for others. The ability to measure accurately specific local interactions permits the study in depth of a region of the protein without the need to be concerned with details of other regions. This is of particular interest in the definition of conformational changes, such as those which follow inhibitor or substrate binding.

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