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High-Resolution Nuclear Magnetic Resonance Studies of the *Lac* Repressor. 1. Assignments of Tyrosine Resonances in the N-Terminal Headpiece†

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ABSTRACT: The DNA binding site of the *lac* repressor protein has been implicated to lie within the N-terminal 51 amino acid fragment termed headpiece (HP-51 or LR-51). High-resolution NMR suggests that isolated HP-51 retains most of the secondary and tertiary structure which it has in the whole repressor. Four of the eight tyrosines of repressor are in HP-51. ^{1}H NMR spectra (360 MHz) over the aromatic region of native HP-51 show that the four tyrosines are nonequivalent with an unusual distribution of chemical shifts. Denaturation leads to loss of these chemical shift differences. Homonuclear decoupling and a two-dimensional autocorrelated spectrum allow unequivocal pairing of resonances from Tyr A at 6.99 and 6.79 ppm, Tyr B at 6.98 and 6.39 ppm, Tyr C at 6.70 and 6.54 ppm, and Tyr D at 6.39 and 6.33 ppm. The 2,6 protons are low field of the 3,5 protons for each Tyr residue. Selective chemical modification with nitration reagents allows assignments of Tyr A to Tyr-47, Tyr B to Tyr-7, Tyr C to Tyr-12, and Tyr D to Tyr-17 in HP-51. All four tyrosines are essential for maintaining the structure of the isolated headpiece, and Tyr-7, -12, and -17 appear to be stacked.

The *lac* repressor (LR) is a regulatory protein of *Escherichia coli* originally isolated by Gilbert & Muller-Hill (1966). Its function in the intact bacterium is to form a complex with the operator region of the *lac* operon, blocking transcription and hence the production of lactose metabolizing enzymes and transport proteins. Allolactose and various galactosides serve as inducers of protein synthesis by binding to the repressor and cause dissociation of the DNA-repressor complex (Jobe & Bourgeois, 1972). Intact repressor has a molecular weight of 154 500 and is a tetramer of four identical subunits, each with a known sequence of 360 amino acids (Beyreuther, 1978; Farabaugh, 1978). The three-dimensional structure of this protein has not been determined. Given the primary sequence, NMR may allow some insight into the structure, and the present studies were undertaken on this basis.

Proteolytic cleavage of the repressor (Geisler & Weber, 1977) permits the preparation of two fragments: the tetrameric "tryptic core" (TC- or LR-60-360), which contains the inducer binding site, and the N-terminal headpiece (HP- or LR-51 or 59), which is monomeric and contains the major portion of the operator (DNA) binding site (Pfahl et al., 1974; Miller et al., 1975, 1977; Barkley et al., 1975). ^{1}H NMR spectroscopy has given the surprising result that the spectra of HP and TC are simply additive, yielding a spectrum identical with that of the whole repressor (Wade-Jardetzky et al., 1979; Buck et al., 1978). This result suggests that the headpiece is a separately mobile domain of the repressor molecule and is consistent with recent low-angle X-ray scattering experiments (Pilz et al., 1980) which demonstrate that HP lies at the ends of a cigar-shaped tetrameric core.

HP contains four of the eight tyrosines and one of the six histidines in the repressor. The tyrosine resonances are nonequivalent with an unusual distribution of chemical shifts. It is the purpose of this paper to report an analysis of the aromatic region of the HP ^{1}H NMR spectrum and the assignment of the tyrosine resonances. In subsequent reports, we will discuss other features of the structure and dynamics of the *lac* repressor and its structured fragments HP and TC, which can be inferred from high-resolution NMR spectroscopy.

Materials and Methods

Lac repressor protein was prepared from cultures of the SQ mutant of *E. coli* as previously described (Matthews et al., 1977). The tryptic core (TC) and headpiece (HP) fragments of LR were prepared according to the procedure of Geisler & Weber (1977). LR, at a protein concentration of 15 mg/mL in 1 M Tris-HCl buffer (pH 7.6), 30% glycerol, and 3×10^{-4} M dithiothreitol, was digested with 1.5% of its weight of DCC-treated trypsin (Sigma Chemical Co.) for 2 h at 20 °C. The trypsin was then inactivated by a 3-fold excess of soybean trypsin inhibitor (Sigma Chemical Co.). Under these conditions, peptide bond hydrolysis at lysyl residue 59 goes to completion, while a partial cleavage occurs at arginyl residue 51. The monomeric HP fragments (residues 1-51 and 1-59) and the tetrameric TC (residues 60-360) were separated by gel filtration at 5 °C on a column of Sephadex G-150 eluted with 0.2 M ammonium bicarbonate and 3×10^{-4} M dithiothreitol. HP was further purified by gel filtration on a column of Sephadex G-50 with the bicarbonate buffer, and HP-51 and HP-59 were then separated by ion-exchange chromatography on a column of CM-Sephadex C-25 and eluted with a gradient

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from 0 to 0.2 M potassium chloride in a pH 7.6, 0.02 M Tris-HCl buffer containing 5% glycerol. Native and denatured HP materials were separated by gel filtration on a column of Sephadex G-50 at high salt concentration—0.2 M, pH 7.8, Tris-HCl buffer, 0.8 M KCl, 1 mM EDTA, and 0.2 mM dithiothreitol. The effectiveness of this column in removing denatured peptide is shown by the spectrum in Figure 1.

Chemical modification of the tyrosine aromatic resonances was carried out by treating native HP in pH 7.9 potassium phosphate buffer with tetranitromethane, introduced into the aqueous protein solution as a 10% solution in Me_2SO. The reaction conditions are given in Table II. Reagent and carrier solvents were subsequently removed by dialysis. Under these conditions, the only product on nitration of tyrosine was 3-nitrotyrosine (Sokolovsky et al., 1966; Snyder et al., 1976). Aggregated protein material appearing as a side product was separated from HP prior to spectroscopic measurement by Sephadex G-50 chromatography as described above.

The extent of Tyr modification was determined by two methods: (a) Sequence analysis of the N-terminal 20 amino acid fragment with Tyr-7, -12, and -17 on a Beckman 890C sequencer and (b) isolation of tyrosine peptide fragments on a peptide map with subsequent spectrophotometric analysis. Each sample to be sequenced (50–150 nmol) was subjected to 20 Edman degradations, using dimethylallylamine buffer in the coupling step. Extractive losses of HP were minimized by the addition of a carrier, 1 mg of re-N-acetylated cytochrome *c*, to the sequencer cup. Phenylthiohydantoin (PTH)–norleucine (50 nmol) was added to the *n*-butyl chloride extracts of the 20 Edman degradations. The PTH derivatives were quantitated by gas–liquid chromatography, with PTH–norleucine being used as an internal standard. In the sequencing experiments, semilog plots of nanomoles of PTH vs. residue number for the hydrophobic residues 4, 6, 9, 10, 13, 15, and 20 were nearly linear. Such plots gave the expected yields for residues 7, 12, and 17 for control samples of native HP, and percentage modifications for the tyrosines were calculated from this calibration curve.

The percent modification at 47 was calculated from the average percent Tyr modification obtained by amino acid analyses and the percent Tyr modification at 7, 12, and 17 obtained from the sequence analysis. For amino acid analysis, the nitrated HP samples were hydrolyzed in 6 M HCl at 110 °C for 20 h in evacuated, sealed tubes since 3-nitrotyrosine is stable to acid hydrolysis (Sokolovsky et al., 1966). The amino acid derivatives in the chemically modified HP samples were identified and quantitated by amino acid analysis on a Durrum D-500 autoanalyzer.

In some cases, the yields of tyrosine at positions 7, 12, and 17 were confirmed by amino acid analysis after back hydrolysis of the PTH's to the free amino acid in acid media (Mendez & Lai, 1975).

The percent modification of nitrated HP samples was also determined by peptide mapping by using the procedures of Katz et al. (1959) and Easley (1965). The modified proteins were dialyzed against 0.05 M ammonium bicarbonate and heat denatured at 80 °C for 10 min. Peptides were generated at 37 °C by three additions of 2% chymotrypsin at 3-h intervals and by two additions of 2% trypsin. The peptides were then separated by two-dimensional chromatography and electrophoresis on full 18.5 × 22.5 in. sheets of Whatman 3MM filter paper, and the amount of 3-nitrotyrosine was determined spectrophotometrically following elution of each spot with 5% formic acid–10% acetic acid.

For NMR studies, HP was concentrated by precipitation at 5 °C by the addition of solid ammonium sulfate to 90% saturation and was extensively dialyzed in Spectrapor 3 membrane tubing (Spectrum Medical Industries, Inc.) against a 0.30 M potassium phosphate buffer in D_2O containing 0.20 M potassium chloride, 1×10^{-4} M dithiothreitol, and 1×10^{-4} M ethylenedinitrilotetraacetic acid, pD 7.9. Solutions with different pD values were prepared by the addition of appropriate amounts of 10 mM DCl or NaOD. Fourier-transform (FT) ^1H NMR spectra were recorded on the modified Bruker HXS-360 spectrometer at the Stanford Magnetic Resonance Laboratory. Chemical shifts were measured relative to external 4,4-dimethyl-4-silapentanesulfonate (DSS) (1% DSS dissolved in D_2O). The two-dimensional autocorrelated spectrum as described by Aue et al. (1976) was taken with 512 points in the t_1 dimension and 2048 points in the t_2 dimension, with 64 averages at each t_1 point; zero filling was then used for Fourier transforms in the t_1 dimension. The delay between experiments was 2.7 s.

Results

Spectra of the aromatic region of native and heat-denatured HP are shown in Figure 1 (B and D). The observed aromatic region is due to histidine at position 29 and the four tyrosines at positions 7, 12, 17, and 47 in the primary sequence. At pD 7.8 and 23 °C, the chemical shifts of the aromatic resonances are 7.81 ppm for the C-2 proton and 6.92 ppm for the C-4 proton of the histidyl residue and 6.99, 6.98, 6.76, 6.70, 6.54, 6.39, and 6.33 ppm, respectively, for eight tyrosyl doublet resonances corresponding to the four tyrosine residues.

The expected chemical shifts of free histidine in zwitterionic form are 7.8 ppm for the C-2 and 7.0 ppm for the C-4 protons. The assignment of the two histidine peaks is therefore unequivocal. The expected shifts for free tyrosine are 7.1 ppm for the 2,6 and 6.8 ppm for the 3,5 protons. Thus the aromatic tyrosyl protons of native HP have higher chemical shifts than the same protons in free tyrosine. In heat-denatured HP, the chemical shifts are 7.03 and 6.77 ppm for the 2,6 and 3,5 tyrosyl protons, respectively. It is apparent that denatured material could easily contribute to the observed intensities of the low-field tyrosyl peaks in native HP, and proper precautions must be taken with HP preparations for any quantitative investigations. The experimental spectrum in Figure 1B indicates that the Sephadex G-50 column procedures described under Materials and Methods are highly effective in removing the bulk of any denatured or aggregated protein. The tyrosine resonances in HP are clearly shown to be chemical shift nonequivalent, with five out of eight doublets shifted upfield relative to the same protons in free tyrosine or the tyrosine residues of denatured HP.

The pairing and distribution of the eight Tyr resonances were established by homonuclear spin decoupling, two-dimensional autocorrelated spectra, and spectrum simulation. The identification of the low-field and upfield components of the individual tyrosine spectra as the 2,6 and 3,5 protons could readily be made from the temperature dependence of the chemical shifts in the course of unfolding which also indicated that no unusual crossing-over occurs (cf. third paper of three in this issue; Wemmer et al., 1981b). For the four tyrosines, labeled A–D (using subscripts L and U for the low-field and upfield components corresponding to the 2,6 and 3,5 protons), the coupling scheme obtained is the following (see Figure 1 and Table I): A_L, A_U, 6.99, 6.76 ppm; B_L, B_U, 6.98, 6.39 ppm; C_L, C_U, 6.70, 6.54 ppm; and D_L, D_U, 6.39, 6.33 ppm. This scheme was initially explored by one-dimensional homonuclear decoupling experiments, but, in the present case, the degen-

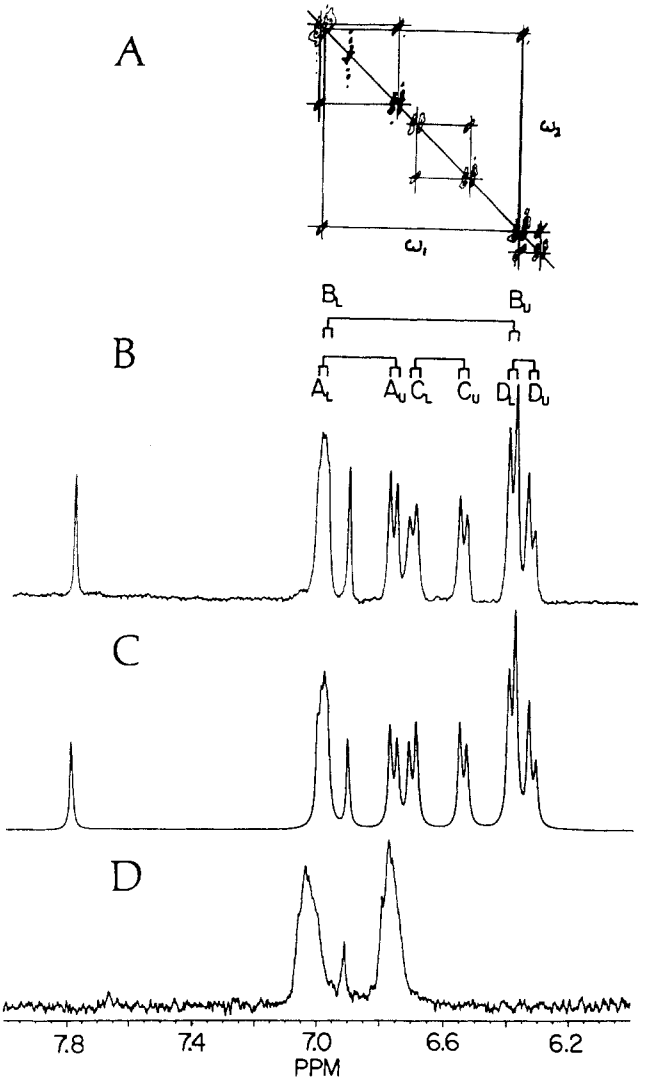

FIGURE 1: (A) Contour plot of two-dimensional autocorrelated 360-MHz spectrum of tyrosine resonances of HP-51 in D_2O solution. ω_1 and ω_2 are both chemical shift frequencies. Cross peaks appear at off-diagonal positions for connected transitions. The tyrosine pairs so delineated are designated A_L,A_U, B_L,B_U, C_L,C_U, and D_L,D_U. (B) FT spectrum (360 MHz) of the aromatic region of a 3 mg/mL sample of HP-51 in 0.3 M potassium phosphate, and 0.2 M KCl buffer in D_2O, pD 7.8, at 23 °C; 10^{-4} M dithiothreitol and 10^{-4} M EDTA were also present in the buffer. The experimental spectrum shown was obtained with a sweep width of ±2500 Hz and 16K points to give a digital resolution of 0.63 Hz/point. The spectrum was obtained with 1024 transients and is line broadened by 1 Hz. (C) Spectrum simulation of the aromatic region of HP-51 by using the decoupling scheme shown in B. Each Tyr pair was simulated, using an AB approximation with the shifts and coupling constants given in Table I. Each line in the simulated spectrum was defined to have a 5-Hz line width. (D) Heat-denatured spectrum of the aromatic region of HP-51 obtained at 70 °C. The 3,5 and 2,6 protons of the four tyrosine residues have approximately identical shifts. The C-2 proton of His is exchanged with the solvent. Experimental conditions are those in B, except for temperature.

eracy of chemical shifts and overlap of resonances, particularly among peaks A_L,B_L and $B_U$$D_L$, prevented a clear interpretation. Moreover, the small chemical shift difference in the case of peaks D_U and D_L (0.06 ppm) makes impossible the selective irradiation of either half of the multiplet at this field strength.

An autocorrelated two-dimensional FT NMR experiment (Aue et al., 1976) was consequently performed. In this two-dimensional FT experiment, the projections in the ω_1 and ω_2 dimensions both correspond to the normal one-dimensional FT spectrum, but cross peaks occur for connected transitions. The spin connectivities of the tyrosine doublets of HP are thus established by merely looking for off-diagonal components in a contour plot of the two-dimensional FT spectrum (Figure 1B) and yield the decoupling scheme listed above. Subsequent spectrum simulation using AB approximation with this decoupling scheme (Figure 1C) gives an excellent agreement with the experimental spectrum of the highly purified HP (Figure 1B).

Table I: Chemical Shifts, Shift Differences, and Assignments for the Tyrosyl Protons of HP

Tyr	δ (ppm) 2,6	δ (ppm) 3,5	Δδ[a] (ppm) 2,6	Δδ[a] (ppm) 3,5	J_{AB} (Hz)	assignment
free Tyr	7.1	6.8				
denatured HP	7.03	6.77				
Tyr A	6.99	6.76	+0.04	+0.01	7.47	47
Tyr B	6.98	6.39	+0.05	+0.38	8.04	7
Tyr C	6.70	6.54	+0.33	+0.23	7.88	12
Tyr D	6.39	6.33	+0.64	+0.44	8.11	17

[a] Given in parts per million as difference between observed shift in intact HP and that observed in denatured HP.

For assignment of the tyrosine resonances to individual residues, the spectra of partially nitrated HP derivatives were compared to those of native HP and interpreted on the basis of amino acid analysis. Under relatively mild conditions of nitration, tyrosines B and D are readily susceptible to chemical modification, and a third tyrosyl residue (C) can be modified under more severe conditions. Representative results of the nitrations are shown in Figure 2. The percent chemical modification as determined by sequence analysis is compared to area loss in the tyrosine region upon chemical modification in Table II.

Under relatively mild conditions of nitration, the largest decrease in tyrosine intensities upon modification is observed at the 6.98- and 6.39-ppm envelopes. The coincidence of decrease at *both* these positions unequivocally suggests that these decreases must arise from modification of Tyr B (Figure 2B). Under more severe conditions of nitration, the intensities of resonances from Tyr B are further decreased. Concomitantly, the intensity of peak D_U appears decreased relative to previous nitration stages or the control spectrum (Figure 2A–C). Finally in Figure 2D, at even more severe conditions of nitration, peaks B and D have been virtually removed. Peaks C_U and C_L appear somewhat more degraded than peaks A_L and A_U.

From the cummulative evidence of nitrated samples under various lengths of incubation times and concentrations of modification reagent, a consistent picture emerges, showing that the order of modification is Tyr B > Tyr D > Tyr C > Tyr A. The His-29 C-4 singlet does not appear affected by nitration since the intensities of the histidine lines and concentrations were identical in several modified and unmodified HP samples.

The percent tyrosine unmodified as determined by sequence analysis or peptide map is compared to area left in the tyrosine region upon chemical modification in Table II for nitration. The percent unmodified by biochemical analysis is directly calculated by taking the percent 3-nitrotyrosine compared to the tyrosine content of the starting material. The percent of area left is calculated for each spectrum, assuming the His C-4 resonance to be one proton in both native and chemically modified HP samples, calculating the number of protons per tyrosine resonance, and expressing as percentage of two protons for each tyrosine doublet. This procedure is not without its potential pitfalls since the relative areas of the tyrosine peaks depend on the purity of the preparation, denatured or partially

Table II: Relative Areas and Modification Extent for Tyrosyl Groups of Headpiece 51

sample	reaction	conditions	relative protons							% area left[a]				% unmodified[a]			
			A_L/B_L	A_U/C_L	C_L	C_U	B_U	D_U	$B_U/D_L/D_U$	B_U	D_U	C_U	A	7	17	12	47
control	nitration		4.13	4.33		1.94	1.92	2.07	6.06	96	103	97	100	100	100	100	100
6X-2	nitration	6X TNM, 2 °C, 2 h	3.72	4.44		1.97	1.38	1.93	5.24	69	97	98	100	70	90	95	100
6X-8	nitration	6X TNM, 2 °C, 8 h	2.57	3.64		1.64	0.48	1.21	2.90	24	61	82	100	20	60	85	100
50X-4	nitration	50X TNM, 2 °C, 4 h	2.35	3.30	1.57	1.66	0.59	1.04	1.63	31	52	79	86	35	50	80	90
60X-4	nitration	60X TNM, 2 °C, 4 h	2.52	3.60	1.72	1.82	0.45	1.12	2.69	23	56	86	94	30	60	90	95
50X-18	nitration	50X TNM, 2 °C, 18 h	1.86	3.34		1.56	0.08	0.60	1.28	4	30	78	89	5	35	80	90
N3[b]	nitration	60X TNM, 2 °C, 8 h	2.05	3.29	1.54	1.04	0.04	0.16	0.52	2	12	52	87	13	36	51	83

[a] Intensities of NMR peaks relative to the His C-4 singlet as one proton obtained by cutting and weighting. With base-line separation between the tyrosine envelopes, the integration errors at worst are estimated at ±8%. The errors of the chemical analysis are estimated to be ±5%. [b] This sample was not purified on Sephadex G-50, and the area measurements are subject to substantial error because of an irregular base line. It is included since it reveals the same order of modification as in other cases. The figures are consistent with the assumption that modification of Tyr-47 shifts the resonances of Tyr-7 and -17, causing additional loss of area of the initial peaks B_U and D_U unrelated to chemical modification of residues 7 and 17.

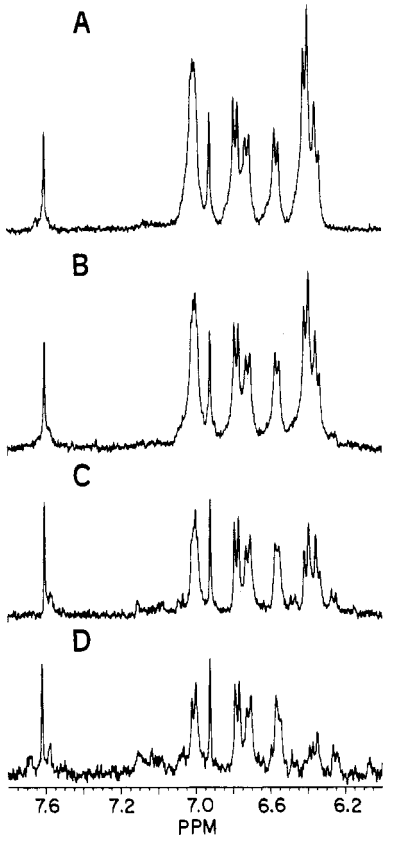

FIGURE 2: ^{1}H NMR spectra (360 MHz) showing the selective nitration of tyrosines in native HP-51 with tetranitromethane. Each spectrum was obtained for HP samples at pD 7.8 and 23 °C with a sweep width of ±2000 Hz and 16K points to give a digital resolution of 0.5 Hz/point. The pulse delay was 3.64 s in each case, with T_1 measured in unmodified headpiece at ~0.5 s. The spectra are line broadened by 0.25 Hz and plotted so that the His C-4 singlet (assumed to represent one proton) has identical height in each spectrum. (A) 1024 scans on a control sample of 0.6 mg of HP-51 in 500 μL of phosphate buffer. (B) 1024 scans on a HP sample reacted with a 6-fold excess of tetranitromethane at 2 °C for 2 h. The largest decrease in intensity is observed at 6.98 and 6.39 ppm due to peaks B_L and B_U, respectively. (C) 1024 scans on a HP sample reacted with a 6-fold excess of TNM at 2 °C for 8 h. Peaks B_L and B_U are essentially removed. Peak D_U is decreased relative to the control. (D) 2000 scans on a HP sample reacted with a 50-fold excess of TNM at 2 °C for 18 h. Peaks B_L, B_U, and D_U are almost completely lost. Peaks C_U and C_L have decreased somewhat more than peaks A_U and A_L. See Table II for quantitative integrations.

denatured material can contribute to peaks A_L, B_L, A_U, and C_L, and overlap of lines can easily lead to errors in integrated intensities (see Table II). The removal of denatured or aggregated protein material, however, leads to a reasonable quantitative analysis. Nitrotyrosine peaks may also interfere with area measurements. In Figure 2, coincidence of a nitrotyrosine peak with the C_U resonance is apparent. However, the nitrotyrosine peaks are readily identified by their pH dependence, and a correction can be applied to the area measurement of the unmodified tyrosine peaks. The appearance of nitrotyrosine doublets in the region 6.3–6.6 ppm indicates that at least some of the HP-containing nitrotyrosine retains its tertiary structure.

The biochemical results for mild modification conditions clearly show that Tyr-7 is the first tyrosine to be modified. Under more severe conditions, Tyr-17, -12, and finally -47 are initiated (Table II). Thus, correlation of the NMR intensities and biochemical analysis allows the assignment of multiplets B and D to Tyr-7 and -17, respectively. A and C thus are immediately assignable as a pair of Tyr-12 and -47. The NMR intensity changes of peaks A and C are less pronounced than those of B and D. Nevertheless, over the course of some 30 samples, and particularly those at severe modification conditions, the Tyr C area is consistently further reduced than that of Tyr A, and the chemical analysis consistently showed that Tyr-47 is less modified than Tyr-12. On this basis, the assignment of Tyr A to Tyr-47 and Tyr C to Tyr-12 can be made.

The uncertainties in the use of chemical modification as a basis for the assignment of NMR resonances in proteins are well-known and have been discussed previously (Roberts & Jardetzky, 1970). However, consistency of the results obtained under different reaction conditions indicates that the reactivity of the tyrosines in HP depends largely on the tertiary structure of the peptide and justifies confidence in the assignments made.

The nonequivalence of the tyrosines in the native preparations constitutes clear evidence for the existence of secondary and/or tertiary structure which is destroyed on heat denaturation (Roberts & Jardetzky, 1970). The fact that the tyrosine peaks appear as moderately well-resolved doublets which may be approximated by AB systems indicates that the aromatic side chains are free to rotate by 180° about the C_β–C_γ bond, at a rate rapid on the chemical shift time scale (Jardetzky, 1970; Campbell et al., 1975).

The aromatic spectrum of a preparation of HP-59 is identical with that for HP-51. This shows that the C-terminal octapeptide of HP-59 is not essential for maintaining structure. In contrast, the peptide fragment containing residues 1–22 and including three of the four tyrosyl residues yields an NMR spectrum indistinguishable from heat-denatured HP (Figure 1), suggesting that some residues beyond position 23 are es-

sential for this purpose. Sequential digestion of HP-51 at the C-terminal region by carboxypeptidase Y suggests that the rate of disappearance of structure as monitored in the aromatic region parallels the rate of release of C-terminal residues measured by the rate of appearance of free tyrosine. Thus, it may be concluded that at least 47 of the 51 residues of HP-51 are essential for maintaining the structure while residues 52–59 are clearly dispensable.

Discussion

Recent low-angle X-ray results (Pilz et al., 1980) have confirmed the NMR findings that HP exists as a separate domain from the tryptic core domain of the intact repressor and that the domains are connected by a flexible hinge area of the polypeptide chain. Geisler & Weber (1977) have suggested that this hinge is localized between residues 50 and 60. Several interesting features of the HP domain are noted in this work. His-29 appears to be at or near the surface of the peptide, both C-2H and C-4H chemical shifts suggesting free access to the solvent. Tyr-47 is very near the flexible hinge that connects the HP domain to the core but is not part of that hinge since its removal results in the disintegration of the structure and since it is accessible to reagents only with some difficulty. The anomalous shifts of the resonances of Tyr-7, -12, and -17 could have several explanations: (1) proximity to negatively charged groups, (2) embedding in a highly hydrophobic environment, and (3) mutual stacking. Tyr-7 and Tyr-12 indeed have charged neighbors Asp-8 and Glu-11 whereas Tyr-17 and Tyr-47 have uncharged neighbors Gln-18 and Asn-46 [for a sequence, see Geisler & Weber (1977)]. Spectra at lower and higher pH indicate some shifting of the tyrosyl resonance positions, but these are generally only on the order of 0.1–0.15 ppm and are much smaller than the observed chemical shift differences of 0.3–0.6 ppm for the various tyrosyl protons. Thus, the nearest-neighbor effect does not account for the extreme upfield shift of the Tyr-17 protons relative to all other protons, for the near identity of the shifts for the 3,5 protons of Tyr-7 and -17, or for the marked difference in the shifts of the 2,6 protons of Tyr-7, -12, and -17. Nearest-neighbor charge effects in random coil peptides are relatively small (Roberts & Jardetzky, 1970); therefore a charge contribution to the observed shift must result from proximity in a well-defined structure. Embedding in a hydrophobic environment appears an unlikely mechanism for Tyr-7 and -17 because of their ready accessibility to tetranitromethane. In other proteins where upfield shifted tyrosine resonances can be associated with a highly hydrophobic environment of the tyrosine residue, the shifts are associated with a relative inaccessibility to reagents (Snyder et al., 1976).

Stacking of the aromatic rings is an attractive alternative to account for the unusual chemical shift positions of Tyr-17, -7, and -12. One is then faced with the choice of an irregular backbone structure or a regularly folded backbone structure which permits the stacking of tyrosine rings. Published CD evidence (Geisler & Weber, 1977) for a mixture of HP-51 and HP-59 suggests the presence of a highly ordered helical structure. However, stacking of Tyr-7, -12, and -17 cannot be achieved in an α helix.

Two alternative helical structures can be advanced to give rise to the stacking of tyrosine rings and unusual chemical shift positions evidenced in the ^{1}H NMR spectrum, the 4.3_{14} δ-helical structure (Chandrasekaran et al., 1979; Donohue, 1953) and the 4.4_{16} π-helical structure (Bamford et al., 1956; Donohue, 1953). The pitch of these helices are 5.16 Å and 5.06 Å, respectively. In both structures, the tyrosine side chains are located on the *same* face of the helical structure and may stack above each other. The possible ring spacings in both structures are in the 6–7-Å range. The order of magnitude for the stacking shifts at 6–7-Å distances can be estimated to be in the range 0.3–0.5 ppm, in agreement with those experimentally observed shifts in the spectrum (Table I). Precise values cannot be given much meaning since additional shift contributions will arise in the structure from the constrained negative charges and side chains in close proximity in the tertiary structure.

The principal interest in helical structures for the 20 N-terminal amino acid residues of HP stems from the fact that this segment contains part of the DNA binding site (Miller et al., 1975), and 6.8 Å is the spacing necessary for the intercalation of the tyrosines into the DNA double helix. Intercalation of tyrosine side chains into DNA as a mechanism for protein–DNA interactions has been previously proposed by Dimicoli & Helene (1974). The present state of the evidence does not warrant an elaborate defense of the helices as a structural feature of the *lac* repressor. Alternative, irregular backbone structures, accounting for the observed shifts and permitting stacking of tyrosine rings and intercalation, are undoubtedly possible. However, the features of the regular structures are consistent with the NMR data and are sufficiently striking, considering the DNA-binding function of the *lac* repressor, to deserve special mention.

To summarize the salient points: (1) HP is not a random coil but a highly structured peptide. (2) Residues 2–47 are required to maintain the HP structure, but residues 1 and 52–59 are not. (3) The structure as a whole has considerable flexibility as shown earlier (Wade-Jardetzky et al., 1979). (4) It constitutes a separate movable domain in the intact repressor. (5) Tyr-7, -12, and -17 in native HP are likely to be stacked to some extent. (6) Tyr-47 does not appear to be stacked from its "normal" chemical shift but appears necessary to maintain structure as suggested by the C-terminal degradation experiments. On the other hand, the chemical modification experiments also show this residue to be relatively inaccessible. (7) The single histidine shows no unusual shifts and appears to be near the surface of the structure, freely mobile and accessible to solvent.

Structural features evident in the aliphatic portion of the ^{1}H NMR spectrum of HP are reported in the following paper in this issue (Ribeiro et al., 1981).

Acknowledgments

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High-Resolution Nuclear Magnetic Resonance Studies of the *Lac* Repressor. 2. Partial Analysis of the Aliphatic Region of the *Lac* Repressor Headpiece Spectrum†

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ABSTRACT: The 360-MHz ^{1}H NMR spectrum of native *lac* repressor headpiece (HP-51 or HP-59) contains a large number (>30%) of aliphatic side-chain methyl and backbone α-CH resonances and three of four aromatic tyrosine multiplet resonances shifted to high-field chemical shift positions, indicating the presence of extensive folded structure. Denaturation leads to loss of the NMR chemical shift differences. Resonance identifications of the 27 methyl-possessing amino acids in HP-59 have been made by using resolution enhancement, double-resonance, and difference spectra. There are three firmly assigned methyl resonances and 21 pairwise identifications of methyl resonances in HP-51. Comparison of HP-51 and HP-59 allows identification of four additional methyl groups in amino acid residues 52–59. The sequence HP-50–59 is not essential to maintain the structure of HP-59, but it is of interest itself as the flexible hinge portion connecting HP to the tetrameric core of whole repressor.

The "headpiece" (HP) is the N-terminal peptide segment of the *lac* repressor, orginally prepared by tryptic cleavage and isolated by Geisler & Weber (1977). Two variants of HP can be prepared and separated chromatographically—one containing residues of 1–59 (HP-59) and the other containing only residues 1–51 (HP-51). In the preceding paper in this issue (Ribeiro et al., 1981) we reported the assignments of the aromatic resonances of the isolated headpiece—i.e., His-29, Tyr-7, Tyr-12, Tyr-17, and Tyr-47—and have suggested that peptides HP-51 and HP-59 have similar native structure. In this communication, we discuss additional structure features which can be deduced from an analysis of the aliphatic region of its ^{1}H NMR spectrum at 360 MHz. The spectrum in the region contains resonances from all 59 (or 51) amino acid residues, including the α-CH and β-CH_2 resonances of the five aromatic amino acids, one His and four Tyr, and the aliphatic amino acids, nine Ala, one Asp, three Asn, three Arg, four Glu, four Gln, two Gly, one Ile, three Leu, four Lys, two Met, two Pro, four Ser, three Thr, and nine Val. Because of the greater complexity, only a partial analysis of the aliphatic spectrum is reported at this time. Nevertheless, assignment of three individual amino acid resonances and the pairwise identifications of 21 of the 27 methyl-containing amino acids have been possible, and several significant features of the structure of the peptide have emerged.

Materials and Methods

HP preparations and spectroscopic measurements were generally carried out as previously described (Ribeiro et al., 1981). Chemical modifications of tyrosine residues in native HP with tetranitromethane as the nitration reagent were also previously described (Ribeiro et al., 1981). Iodination of the tyrosine residues was carried out by treating native HP with iodine–potassium iodide reagent at temperatures of 5, 25, and 37 °C and for reaction times of 0.5–5 h. Varying degrees of modification of the four tyrosine residues in HP are seen in

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