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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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the ^1H NMR spectrum (Ribeiro et al., 1981). In Figure 4A, 0.94 mg of native HP in 500 μL of potassium phosphate buffer was reacted at 5 °C for 5 h with 25 μL of 0.04 M iodine–0.40 M potassium iodide solution. A 10-μL aliquot of 0.1 M sodium thiosulfate was used to stop the reaction. These iodination procedures lead to 3,5-diiodotyrosine derivatives (Koshland et al., 1963; Sherman & Kassel, 1968; Fraenkel-Conrat, 1950) of HP.

HP-50, residues 1–50, was prepared by digestion of HP-59 with a mixture of carboxypeptidases A and B. A solution of HP containing 1.45 mg of protein/mL in 1 M sodium chloride–0.2 M ammonium bicarbonate buffer, pH 8.35, was digested at 37 °C for 3.5 h with a mixture of DFP-treated carboxypeptidases A and B (Sigma Chemical Co.) by using 3% of its weight for A and 0.3% for B. The resultant HP-50 was separated from the carboxypeptidases by gel filtraton at 5 °C on a column of Sephadex G-50, eluted with 0.2 M ammonium bicarbonate and 3×10^{-4} M dithiothreitol, concentrated in a 10-mL Amicon ultrafiltration cell using a UM2 membrane, and dialyzed against a pH 7.9 buffer in D_2O. The amino acid analysis was performed on a Durrum D-500 autoanalyzer after 6 N HCl hydrolysis.

HP-2–59 (residues 2–59) was prepared by proteolysis of HP-59 with aminopeptidase M (Wachemuth et al., 1966). A solution of HP (2 mg of protein/mL) in 0.06 M potassium phosphate buffer and 0.05 M potassium chloride, pH 7.9, was digested with 2% of its weight of aminopeptidase M (Sigma Chemical Co.) at room temperature for 25 h. HP-2–59 was purified on a column of Sephadex G-50. Amino-terminal analysis was performed by using the peptide program of the Beckman 890C sequencer. The first five residues were identified and quantitated by gas–liquid chromatography.

The peptide including residues 12–36 was generated by cleavage of HP at Glu-11 and Glu-36 with an endoprotease highly specific for glutamyl residues (Houmard & Drapeau, 1972; Drapeau et al., 1972). A solution of HP-51 (1.47 mg of protein/mL) in 0.2 M ammonium bicarbonate was digested with 2% of its weight of Staphylococcal protease from *S. aureus* V8 (Miles Laboratories, Inc.) at room temperature for 17 h. The peptide encompassing residues 12–36 was purified on a column of Sephadex G-50.

Fourier-transform (FT) ^1H NMR spectra were recorded as previously described (Ribeiro et al., 1981) with the following additional procedures. For improvement of the spectral resolution in the aliphatic region, digital filtering techniques (Ernst, 1966) were applied to the free induction decays. A double exponential giving negative Lorentzian and positive Gaussian broadening (Ferrige & Lindon, 1978) with appropriate choice of parameters allows resolution enhancement while retaining reasonable signal-to-noise ratio. NOE difference spectra (Richarz & Wuthrich, 1978) were obtained in the following manner: four scans were taken in which the NOE was built up by irradiation of a selected resonance for 2 s; then four scans were taken with the decoupler at an off-resonance position. This loop was then cycled to improve the signal-to-noise ratio. After accumulation was completed, each FID was Fourier transformed and the difference spectrum was calculated.

Results

Spectra of the aliphatic region of native HP-51, HP-59, and heat-denatured HP-51 are shown in Figure 1. A comparison of the spectra of the native and denatured peptide immediately reveals extensive structural features in the native form. It is known that the NMR spectra of unfolded peptides and proteins are closely approximated by the sum of the spectra of the

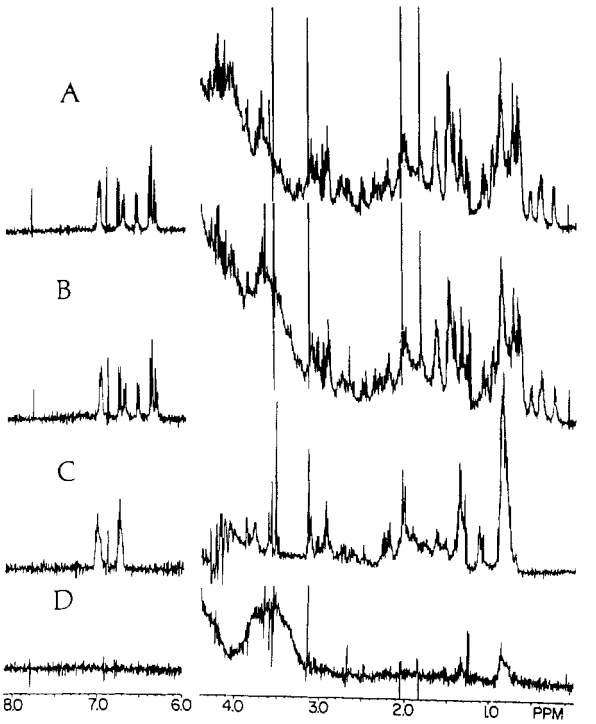

FIGURE 1: Resolution-enhanced 360-MHz ^1H NMR spectra of the *lac* repressor headpiece. The FID's were obtained at a sweep width of ±2000 Hz, 16K points for a digital resolution of 0.5 Hz/point, and resolution enhanced before Fourier transformation. The singlets at 3.14 and 3.55 ppm arise from dithiothreitol and EDTA in the buffer solution. (A) Native HP-51 (3 mg/mL) at pD 7.8 and 23 °C in phosphate buffer in D_2O, 720 scans. (B) Native HP-59 (1 mg/mL) at pD 7.8 and 23 °C in phosphate buffer in D_2O, 1700 scans. (C) Heat-denatured HP-51 (2 mg/mL) at pD 7.8 and 75 °C in phosphate buffer in D_2O, 500 scans. The HOD peak has shifted to ~4.2 ppm at 75 °C and has been nulled by using a burst of radiofrequency power from a decoupling channel. (D) Difference spectrum of (B) and (A), revealing methyl groups arising from the C-terminal octapeptide of HP-59. The broad peak at 3.9 ppm probably stems from differences in the wings of the residual peak from solvent water and is not significant. The sharp peak at 3.19 comes from a buffer component.

constituent amino acids whereas additional chemical shift nonequivalence arises from the formation of secondary and tertiary structure (Roberts & Jardetzky, 1970; Phillips, 1973). Particularly noteworthy are the following features in Figure 1A,B: (a) an upfield shift of about 30–40% of the α-CH resonances, normally found in peptides about 4.5–5 ppm into the region of 3.8–4.2 ppm—such a shift is usually associated with extensive secondary structure, notably helix formation (Markley et al., 1967); (b) four methyls in the high field region 0.0–0.5 ppm in the native structure—these resonances merge with the main methyl peaks upon denaturation (Figure 1C); (c) a clear chemical shift nonequivalence of the tyrosine resonances which appear as well-resolved doublets in native material and become equivalent in the denatured material (Figure 1C); (d) a methionine SCH_3 singlet at an upfield shift of 1.83 ppm in native material and a random coil chemical shift of 2.0 ppm in denatured HP; (e) the nonequivalence of alanyl, threonyl, valyl, leucyl, or isoleucyl methyl peaks between 1.6 and 0.6 ppm.

The NMR spectra of HP-51 and HP-59 in both the aromatic and aliphatic regions (Figure 1A,B) appear identical except for resonances arising from the differences in amino acid content, i.e., residues 52–59 consisting of the sequence -Val-Ala-Gln-Gln-Leu-Ala-Gly-Lys. This significant finding suggests that the basic structure of the two peptides is the same and the C-terminal octapeptide is not essential to maintain its integrity. This allows experiments to be carried out on

Table I: Identification and Assignments for Aliphatic Groups of *lac* Repressor Headpiece[a]

amino acid	no. of residues	sequence positions	CH_3 δ (ppm)[b]	side chain δ (ppm) γ	β	α	possible assignment
Ala	9	10, 13, 27,	1.635			4.03	Ala
		32, 40,	1.512			4.23	Ala
		41, 43,	1.476			4.20	Ala
		53, 57	1.426			3.96	Ala
			1.357			4.84	Ala
			1.278			4.20	Ala
			1.246[c]			4.03[c]	Ala-53/Ala-57
Thr	3	5, 19, 34	0.961		4.08		Thr
			0.977		4.26		Thr
			1.338		4.16		Thr
Val	9	4, 9, 15,	1.042, 1.085		2.09	4.29	Val
		20, 23,	0.88, 0.93		2.36		Val
		24, 30,	0.85, 0.923		2.23	4.42	Val
		38, 52	0.878, 0.900		2.03	4.11	Val
			0.885, 0.745		1.80	4.13	Val
			0.640, 0.734		1.86		Val
			0.630, 0.775		2.12	4.01	Val
			0.521, 0.65		2.03	3.66	Val
			~0.7–0.9[c]				Val-52
Ile	1	48	0.672 (δ)	1.35	1.53	4.22	Ile-48
			0.718 (γ)				
Leu	3	6, 45, 56	0.246, 0.683	1.64	1.44	4.56	Leu-6 or Leu-45
			0.388, 0.417	1.18	1.08		Leu-45 or Leu-6
			0.7–0.8[c]	~1.3	~1.3		Leu-56
Met	2	1, 42	2.045[d]				Met-1
			1.828[d]				Met-42

[a] Data for a 5 mg/mL sample of HP-51 or 2 mg/mL sample of HP-59 in D_2O and phosphate buffer at 23 °C, referenced to external DSS. [b] The random coil positions of methyl resonances of proteins are the following: Ala, ~1.4 ppm; Thr, 1.2 ppm; Leu, 0.9 ppm; Met, 2.0 ppm; Ile, 0.9 ppm for δ and 1.1 ppm for γ CH_3; Val, 0.9 ppm (Roberts & Jardetzky, 1970). [c] Identified by difference spectrum of HP-51 and HP-59 (Figure 1D). [d] Assigned by enzymatic cleavage or iodination of Met-1 (see text).

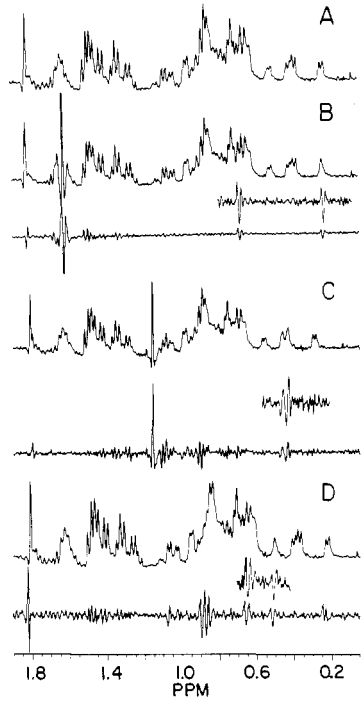

FIGURE 2: Resolution-enhanced 360-MHz ^1H NMR spectrum between 0 and 1.9 ppm, showing the decoupling of $CH(CH_3)_2$ spin systems in native HP-51 at 5 mg/mL, pD 7.8, and 23 °C in phosphate buffer in D_2O. The spectra were obtained at a sweep width of ±2500 Hz, 16K points for a digital resolution of 0.63 Hz/point, and are the sum of 512 transients. (A) Off-resonance spin decoupling at −0.1 ppm. (B) Spectrum with spin decoupling at 1.63 ppm and difference spectrum of (B) from (A). (C) Spectrum with spin decoupling at 1.18 ppm and difference spectrum of (A) from (C). (D) Spectrum with spin decoupler at 2.0 ppm and difference spectrum of (D) from (A).

either peptide and valid resonance identifications to be made for both.

The nine Ala, three Thr, nine Val, one Ile, three Leu, and two Met residues in HP-59 total to 40 methyl groups arising from residues at the sequence positions in Table I. Among these, the methionine SCH_3 groups give singlet resonances at 2.05 and 1.83 ppm. These methionine resonances are distinguished by comparison to the ^1H NMR spectrum of HP-2–59 (see Materials and Methods), in which the N-terminal methionine has been removed by aminopeptidase. The loss of Met-1 does not destroy the structure but leads to loss of the 2.05-ppm singlet, thus assigning the upfield shifted Met at 1.83 ppm as the Met-42 SCH_3 resonance. Removal of residues 1–11 and 37–51 by Staphylococcal protease, however, results in a denatured spectrum and complete loss of structure.

Homonuclear double resonance experiments were performed to determine the nature of the methyl groups between 1.6 and 0 ppm. It is obviously necessary here to distinguish linear side chains such as alanine or threonine from branched side chains such as valine or leucine. In particular, care must be exercised to differentiate the resonance of a valine or leucine $CH(CH_3)_2$ system from the coincidental overlap of CH resonances from two separate $CH(CH_3)$ systems. The difference between the decoupling frequency and a decoupled resonance allows this distinction by giving an indication of whether the $CH(CH_3)$ is an α CH, β CH, or γ CH. Decoupling was carried out starting at the 4.8-ppm region under the water signal and slowly stepped through the 4.8–3.5 ppm α-CH region, then the 3.5–2.0-ppm β-CH region, and finally the 2.0–0-ppm "methyl" region of the spectrum. The results presented are the outcome of over 160 decoupling spectra of which only a few examples will be shown.

The resolved high-field methyl doublets between 0.5 and 0.2 ppm are of particular interest (Figure 2). Figure 2B reveals that a proton group at ~1.65 ppm and the methyl doublet at 0.23 ppm are part of one spin system: subtracting the off-resonance spectrum from the decoupled spectrum

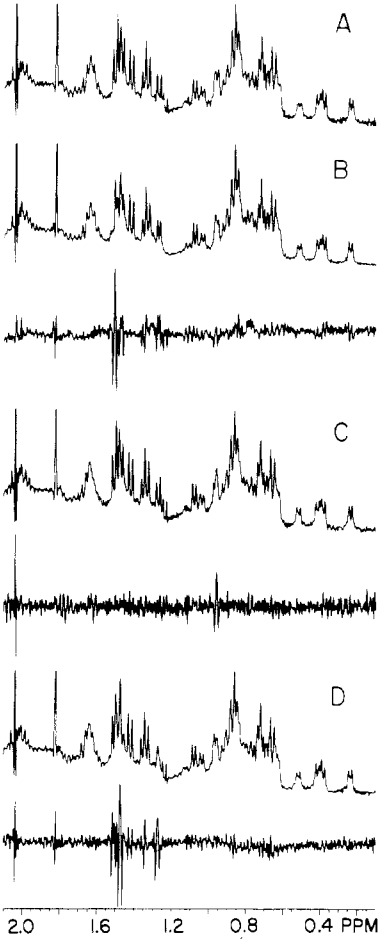

FIGURE 3: Resolution-enhanced 360-MHz ^{1}H NMR spectrum between 0.1 and 2.0 ppm showing the decoupling of $CH(CH_3)$ spin systems in native HP-51; conditions are as shown in Figure 2. (A) Off-resonance spin decoupling at −0.1 ppm. (B) Spectrum with spin decoupling at 4.23 ppm and difference spectrum of (A) and (B). The doublet at 1.512 ppm is seen to individually collapse to a singlet. (C) Spin decoupling at 4.09 ppm and difference spectrum of (A) from (C). The 0.961-ppm doublet individually collapses to a singlet. (D) Spin decoupling at 4.20 ppm and difference spectrum of (A) from (D). Two doublets at 1.278 and 1.476 ppm simultaneously collapse to singlets. However, since the decoupling frequency is clearly in the α-CH region, this cannot be due to a branched side-chain amino acid but must be due to the coincidental overlap of two α-CH resonances.

(Figure 2B) reveals that a second methyl doublet of 0.65 ppm also decouples here, and these three resonances come from the same amino acid residue. Similarly, irradiation at 2.02 ppm decouples the methyl doublet at 0.511 ppm and a hidden methyl doublet at ~0.64 ppm (Figure 2D). The remaining two resolved methyl doublet resonances at 0.378 and 0.407 ppm are decoupled only upon irradiation at the unusually high-field position of 1.18 ppm (Figure 2C). The isopropyl groups implicated in these three cases can only arise from leucyl or valyl residues.

Double-resonance irradiation at positions between 4.8 and 3.5 ppm caused the collapse of a total of nine methyl doublet resonances to singlets. These changes were clearly detected with difference spectra (Figure 3). For example, the doublets at 1.512 and 0.961 ppm are individually collapsed when decoupling at 4.23 and 4.09 ppm, respectively (Figure 3B,C).

Irradiation at 4.20 ppm, on the other hand, led to the simultaneous collapse of two methyl doublets at 1.476 and 1.278 ppm. This result is, however, clearly due to the coincidental overlap of two separate CH systems since the decoupling frequency is in the α-CH region of the ^{1}H NMR spectrum and a slow stepping of the decoupler allows conditions for a preferable collapse of the 1.476 ppm doublet with little collapse of the 1.278-ppm doublet. Similar phenomena are seen for the doublets at 1.63 and 1.25 ppm which decouple at 4.03 ppm. The AX_3-like relationships thus observed can only arise in Ala or Thr side chains, and the nine methyl doublets accordingly correspond to the nine of the ten (seven Ala and three Thr) linear methyl chains in HP-51. Threonine and alanine are also distinguishable from their known random coil positions, with alanyl methyls generally appearing at 1.4 ppm and threonyl methyls at 1.2 ppm. Upon heat denaturation, the 0.951-, 0.967-, and 1.338-ppm methyl resonances are found to shift to 1.2 ppm and are consequently identified as threonines, while the remaining AX_3 methyl doublets shift to 1.4 ppm and are consequently identified as alanines. The behavior of the methyl and aromatic resonances with temperature is described in the next paper (Wemmer et al., 1981).

Additional methyl resonances in HP-51 come from the single Ile, two Leu, and eight Val residues. An Ile gives rise to one methyl doublet and one methyl triplet. Irradiation at 1.28 ppm gives the only methyl triplet–singlet difference spectrum at 0.662 ppm, thus uniquely identifying this methyl triplet as arising from the δ-CH_3 of Ile-48. Similarly, irradiation at ~1.53 ppm causes the collapse of one methyl doublet at 0.718 ppm which is thus identified as the Ile-48 γ-CH_3.

A total of ten methyl pairs were found to simultaneously collapse to singlets when decoupling, respectively, at 2.36, 2.23, 2.12, 2.09, 2.03, 2.03, 1.86, 1.80, 1.63, and 1.18 ppm. These arise from the two Leu and eight Val residues of HP-51.

Irradiation of valyl β-CH protons should give changes in both the methyl and α-CH region while irradiation of leucyl γ-CH should induce a change in the appearance of the leucyl β protons and no changes in the α-CH region. Irradiation at 1.13 and 1.63 ppm (Figure 2A, B) revealed difference spectra with no changes in the α-CH region and noticeable differences in the 1–2-ppm region, thus identifying the 0.246, 0.683 ppm and 0.388, 0.417 ppm high-field doublet pairs as arising from the Leu residues at positions 6 and 45. Decoupling at the other eight frequencies induced visible changes in the α-CH region accompanying the methyl pair collapse, and these resonances are thus identified as valyl residues. One of these corresponds to the high-field shifted methyl pair at 0.521 and 0.65 ppm (Figure 2C).

Additional methyl groups are present in HP-59. The difference spectrum between HP-51 and HP-59 (Figure 1D) reveals a prominent envelope between 0.7 and 0.9 ppm, a sharp doublet at 1.25 ppm, and a multiplet resonance at 1.32 ppm. The 0.7–0.9-ppm envelope clearly is composed from the Val-52 and Leu-56 methyl groups. The sharp doublet corresponds to at least one of the two Ala CH_3 at positions 53 or 57. The 1.3-ppm envelope presumably arises from the Leu-56 β and γ protons.

Chemical modification of the tyrosine rings with iodine (see Materials and Methods) or tetranitromethane (Ribeiro et al., 1981) has a selective and dramatic effect on the appearance of the high-field methyl resonances. After almost complete iodination of Tyr-7 and -17, most of the aliphatic and aromatic regions retain the general features seen for native HP, showing that the HP structure remains intact and the protein is not denatured (Figure 4). At this stage of modification, however, the 0.23-, 0.378-, and 0.407-ppm leucyl methyl doublets are conspicuously shifted to low-fields while the 0.52-ppm valyl doublet shifts *upfield*. Further iodination of Tyr-12 and Tyr-47 destroys the HP structure, and all the high-field methyls merge into the large methyl envelope at 0.8 ppm seen

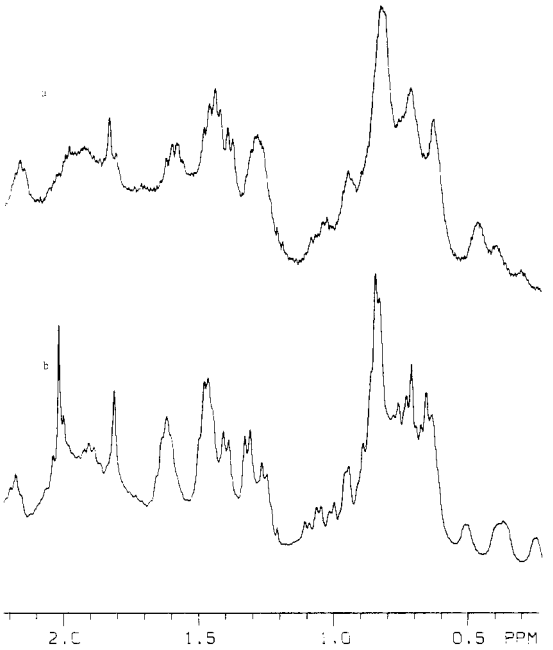

FIGURE 4: Effect of tyrosine iodination on aliphatic groups in the *lac* repressor headpiece: (a) iodinated and (b) control. In (a), the Met-1 SCH_3 singlet has disappeared from the spectrum and the ~0.5-ppm methyl has shifted upfield while the 0.38-, 0.42-ppm methyls and 0.23-ppm methyl has shifted downfield. Some other small changes are seen in the 0.6–1.6-ppm region.

in denatured HP. Smaller differences are notable in the Ala–Thr resonances between 1.2 and 1.5 ppm. A side reaction of iodine involving selective oxidation of Met-1 is also evident in the spectrum since the peak of Met-1 is completely absent in iodinated HP.

Less pronounced but similar changes are seen in the high-field methyl region upon nitration (Ribeiro et al., 1981). The absence of extensive line-shape changes elsewhere in the spectrum strongly suggests that the effects of chemically modifying Tyr-7 and -17 on the leucyl and valyl residues represented by the high-field methyls are a direct effect of the ring modification rather than transmitted by an extensive conformational change. It is therefore very likely that the two leucyl and the high-field valyl (0.51 ppm) are in the vicinity of the tyrosine rings in the native structure. The primary sequence of HP with leucine at positions 6 and 45 already suggests that these residues may be near Tyr-7 and -47 in the folded structure. Val-4, -9, -15, and -20 are similarly candidates for the high-field valyl which shifts upon modification of Tyr-7 and -17.

For exploration of the possibilities of interaction between tyrosine rings and methyl resonances, intramolecular NOE experiments were carried out (Figure 5). Irradiation of the 3,5-proton envelope of Tyr-47 and -7 leads to a specific negative NOE for the leucyl methyl pairs at 0.38, 0.41 and 0.23, 0.67 ppm (Figure 5B). Irradiation at the 2,6 protons of Tyr-47 alone, however, leads to a negative NOE only at the 0.38- and 0.41-ppm leucyl methyls (Figure 5D). Irradiation at the 2,6 protons of Tyr-7, on the other hand, induces an NOE only at the 0.23- and 0.67-ppm positions and not at the 0.38- and 0.41-ppm positions. As a control, the His-29 C-4 proton was also irradiated, and no significant changes are seen in the methyl region of the spectrum (Figure 5E). Furthermore, the reverse experiment, irradiation in the methyl region, was carried out. Figure 2F shows that irradiation at the leucyl methyl at 0.38 ppm induces a negative NOE for tyrosine resonances at 7.01 and 6.77 ppm, corresponding to the 2,6 and

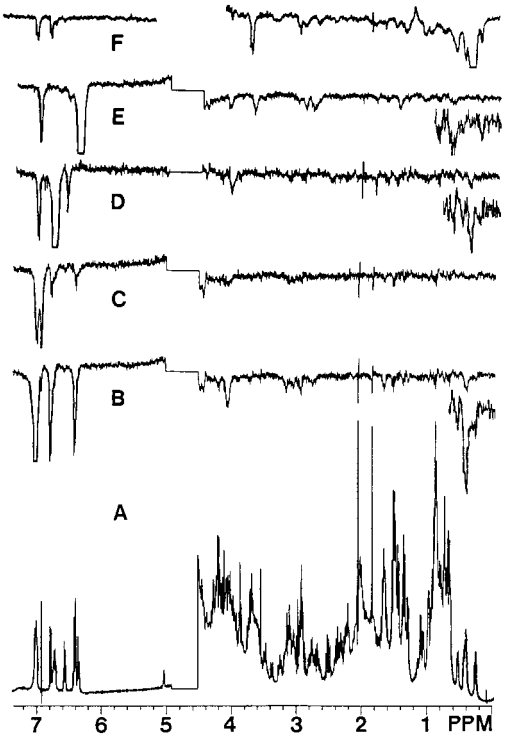

FIGURE 5: ^1H NMR spectra (360 MHz) of the *lac* repressor headpiece at 3 mg/mL in phosphate buffer in D_2O, pD 7.8, and 23 °C. (A) Reference Fourier-transform spectrum with decoupling frequency off resonance obtained at a sweep width ±2500 Hz, 16K points. (B) NOE difference spectrum obtained with presaturation of the 2,6 protons of Tyr-7 and Tyr-47 at 7.0 ppm for a time τ = 2.0 s. The high-field methyls at 0.41, 0.38, and 0.23 ppm have visible negative NOE. The peaks at 6.77 and 6.39 ppm correspond to the 3,5 protons of Tyr-47 and Tyr-7, respectively. (C) NOE difference spectrum obtained with presaturation of the His C-4 proton at 6.91 ppm for τ = 2 s. No significant enhancements appear in the aliphatic region. The tyrosine 3,5 resonances are indirectly enhanced by leakage of rf power to the tyrosine 2,6 protons. (D) NOE difference spectrum obtained with presaturation of the Tyr-47 3,5 protons at 6.77 ppm. The high-field methyls at 0.38, 0.41 ppm show a significant enhancement. The peaks at 7.01 correspond to the Tyr-47 2,6 protons; rf power leaks to the 2,6 protons of Tyr-12 at 6.71 ppm, leading to an enhancement for 3,5 protons of Tyr-12 at 6.55 ppm. (E) NOE difference spectrum obtained with presaturation of 6.39-ppm envelope containing the 3,5 protons of Tyr-7 and 2,6 protons of Tyr-17. The 0.23 ppm high-field methyl shows a small enhancement. The peaks at 6.98 ppm correspond to the 2,6 protons of Tyr-17. (F) NOE difference spectrum obtained with presaturation of the high-field methyl doublet at 0.37 ppm. Significant NOE's are observed for the 6.77- and 7.01-ppm tyrosine doublets corresponding to the 3,5 and 2,6 protons of Tyr-47.

3,5 protons of Tyr-47. The experiments thus verify the proximity of the two leucyl groups to tyrosine resonances suggested by their chemical shift positions and chemical modification.

Discussion

Recent low-angle X-ray scattering results (Pilz et al., 1980), high-resolution NMR spectroscopy (Wade-Jardetzky et al., 1979; Buck et al., 1978), and other physical studies (Barkley et al., 1975; Geisler & Weber, 1977) have given the general picture that the *lac* repressor protein consists of a cigar-shaped tetrameric core domain (TC) composed of amino acids 60–360 and a distinct headpiece domain (HP) composed of amino acids 1–51, connected by a hinge region between residues 50 and 60 (Geisler & Weber, 1977). HP is necessary for the DNA binding of the *lac* repressor and has been shown to bind to nonoperator DNA (Geisler & Weber, 1977, Jovin et al., 1977) and to interact with the *lac* operator region (Ogata and Gilbert, 1978).

In the preceding paper of this issue, we considered the aromatic region of the ^1H NMR spectrum of HP-51 and suggested that Tyr-7, -12, and -17 of HP may stack while Tyr-47 is buried (Ribeiro et al., 1981). Furthermore, a peptide fragment containing residues 1–22 yielded a denatured aromatic spectrum, showing that residues beyond position 23 are essential for native HP structure (Ribeiro et al., 1981).

The identification of the methyl resonances in HP (Table I) add the following information: (1) Each of the two leucines at positions 6 and 45 are in the vicinity of one of the two tyrosines (7 or 47) in the folded HP structure. This is suggested by the appearance of these methyls at high-field positions, their shift with modification of tyrosines, and the observation of an intramolecular NOE. It is unclear at this time whether proximity arises from primary sequence, i.e., Leu-6 near Tyr-7, or from folding, i.e., Leu-6 near Tyr-47. (2) At least three valyl methyl pairs have chemical shifts >0.3 ppm upfield from the random coil position of 0.9 ppm. Likely candidates are valyl-4, -9, -15, and -20 in the vicinity of Tyr-7, -12, and -17. (3) The methyl groups of Ile-48 appear ~0.4 ppm upfield shifted from their random coil positions. (4) Two threonine methyls at 0.961 and 0.977 ppm are substantially upfield shifted, and one threonine at 1.338 ppm is low field shifted from the random coil position of 1.2 ppm. The low-field Thr shift may reflect a local hydrogen bond or salt bridge. A likely candidate would be Thr-34 located between Lys-33 and Arg-35, allowing interaction between the Thr-OH and the neighboring side-chain NH's. The remaining upfield shifted Thr methyls then would arise from Thr-5 and Thr-19. (5) At least two alanyl groups are upfield shifted from the random coil position of 1.4 ppm. (6) The Met-1-SCH_3 at the N terminus is readily oxidized by iodination under mild conditions, has a "normal" shift of ~2.0 ppm, and appears exposed to solvent. Met-42, on the other hand, must be buried since its SCH_3 group is upfield shifted, and it is not modified until the destruction of the tertiary structure. (7) Among the 23 methyl-containing amino acids in HP-51, 15 α-CH resonances are identified in Table I. Seven α-CH resonances are located upfield of 4.15 ppm, and ten are located upfield of 4.25 ppm. The backbone protons of these residues reflect extensive secondary structure, similar to shifts seen in helix formation (Markley et al., 1967).

The overall picture of the structure of the HP domain of the *lac* repressor arrived at by the NMR studies at this point then is the following: (a) An N-terminal tripeptide fragment of Met-1–Lys-2–Pro-3 is most likely accessible to solvent. This N-terminal portion is highly mobile as in other proteins (De Marco et al., 1977), but in HP the segmental mobility does not extend very far down the chain. (b) An extensively structured portion between residues 4 and 20 consists of Val-4–Thr-5–Leu-6–Tyr-7–Asp-8–Val-9–Ala-10–Glu-11–Tyr-12–Ala-13–Gly-14–Val-15–Ser-16–Tyr-17–Gln-18–Thr-19–Val-20. Twelve of the 17 amino acids in this region are probed by the NMR studies in the methyl and aromatic regions. There is reasonable evidence that the bulk of the side chains from this portion contribute to upfield shifted resonances and removal of amino acids 1–11 destroys the structure. It is unclear at this time whether this portion is folded into an irregular or regular structure. Possible models for regular structure which also allow stacking of Tyr-7, Tyr-12, and Tyr-17 include the δ and π helices previously discussed (Chandrasekaran et al., 1979; Ribeiro et al., 1981). (c) There is a relatively unexplored portion between Ser-21 and Ala-41 [see Geisler & Weber (1977) for sequence]. Some clues exist that suggest no unusual folding occurs in this portion. For example, His-29 must be near the surface of the protein, freely mobile, and accessible to solvent as judged by its normal chemical shift and narrow line width (Ribeiro et al., 1981). Valyl groups at 20, 23, 24, 30, and 38 could easily account for the bulk of valyl methyl resonances at "normal" positions of ~0.9 ppm. Alanine residues 27, 32, 40, and 41 may also account for the "normal" Ala resonances at ~1.4 ppm. Thr-34 may give rise to the low-field shifted resonance at 1.338 ppm as stated above. Thus present evidence suggests that the side chains in this portion may not themselves be in unusual tertiary structure, but as previously stated (Ribeiro et al., 1981) some residues in this portion appear necessary for the HP structure. (d) A buried portion consists of Met-42–Ala-43–Glu-44–Leu-45–Tyr-47–Ile-48. As we have seen, the methyl resonances of Met-42, Leu-45, and Ile-48 are upfield shifted. Both Met-42 and Tyr-47 are inaccessible to chemical modification agents. Removal of Tyr-47 parallels destruction of HP structure (Ribeiro et al., 1981). (e) A C-terminal undecapeptide fragment of Pro-49–Asn-50–Arg-51–Val-52–Ala-53–Gln-54–Gln-55–Leu-56–Ala-57–Gly-58–Lys-59 has been implied to constitute the flexible hinge connecting the HP domain to the tryptic core of whole repressor (Geisler & Weber, 1977). The spectra for HP-50, HP-51, and HP-59 are identical except for the differences in amino acid content, and thus Arg-51–Lys-59 are clearly not essential to the folded structure in HP.

The three-dimensional structure of both HP and whole respressor are not yet available from high-resolution X-ray studies. The present NMR studies illustrate our attempts to feel one's way about a protein of unknown structure. Further analysis of the HP spectrum in other regions and extension of the assignments will make it possible to establish other characteristics of the HP structure, to interpret DNA binding studies, and to understand structural transitions.

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High-Resolution Nuclear Magnetic Resonance Studies of the *Lac* Repressor. 3. Unfolding of the *Lac* Repressor Headpiece†

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ABSTRACT: At temperatures below 20 °C, the *lac* repressor headpiece (N-terminal amino acids 1–51) has a well-defined structure which is independent of ionic strength. Its unfolding with increasing temperature proceeds gradually with a characteristic transition temperature which depends on ionic strength. Unfolding has been studied by using NMR and CD. Shifts of several methyl and all of the tyrosyl resonances can be followed, allowing a detailed analysis of the temperature denaturation. At high ionic strength (1 M), the unfolding is complete at 85 °C, while at low ionic strength (0.01 M), it is complete by 65 °C. Native and partially unfolded structures are in rapid exchange during the unfolding, and the process appears completely reversible at all ionic strengths.

The isolated "headpiece" (HP) of the *lac* repressor, containing the 51 N-terminal residues (Geisler & Weber, 1977), has been shown to possess extensive structure and considerable flexibility (Wade-Jardetzky et al., 1979; Ribeiro et al., 1981). This small single-chain polypeptide has no disulfide bridges, making it particularly interesting for the study of protein folding. Changes of the structure with temperature were followed by CD and ^{1}H NMR. Unlike most globular proteins, this protein fragment unfolds in a gradual and continuous manner and in a completely reversible fashion. The temperature at which unfolding is complete is quite dependent upon ionic strength of the solution. Assignments of some of the resonances in the ^{1}H NMR spectrum of this protein to particular residues allow us to develop a model of this transition.

Materials and Methods

Preparations of HP were carried out as described previously (Ribeiro et al., 1981). The standard phosphate buffer at ionic strength ($\Gamma/2$) = 1 contained 260 mM K_2HPO_4, 40 mM KH_2PO_4, 200 mM KCl, 10^{-4} M dithiothreitol, and 10^{-4} M ethylenedinitriloacetic acid. Ionic strength was adjusted by dilution from $\Gamma/2 = 1$ to $\Gamma/2 = 0.01$. NMR experiments were performed on the 360-MHz spectrometer at the Stanford Magnetic Resonance Laboratory. CD measurements were made on a Jasco J-40 auto recording spectropolarimeter at Stanford. Temperature was measured and held to ±1 °C.

Results

The overall unfolding can be monitored by following the CD and NMR spectra as a function of temperature. As secondary and tertiary structures are disrupted, the ellipticity in the CD spectrum is reduced. This is shown for HP at high ionic strength in a D_2O solution in Figure 1. The CD data are consistent with an initial structure which is primarily a helix converting to a random coil. Figure 2 shows the 360-MHz ^{1}H NMR spectra of the HP in 0.3 M phosphate buffer at three temperatures (20, 60, and 80 °C). The low-temperature spectrum represents the native structure and the high-temperature spectrum the completely unfolded form, which is well approximated by a sum of the spectra of the constituent amino acids (Roberts & Jardetzky, 1970). The intermediate spectrum, taken at a temperature near the midpoint of the transition, does not show a mixture of peaks for the folded and unfolded forms but rather peaks with positions intermediate to them. This indicates that the folded and unfolded forms and possibly several partially folded forms are in rapid exchange throughout the temperature range. This is in sharp contrast to the characteristic finding of slow exchange between folded and unfolded forms for many globular proteins near the transition point (Baldwin, 1975).

There is nothing unusual about the transition as monitored by CD. However, NMR provides a method for distinguishing between slow and rapid exchange between folded and unfolded forms and for a more detailed study of the unfolding process. When lines which are assigned to particular residues in the molecule are followed, local and global changes can be distinguished. Large shifts of peaks with temperature are particularly obvious in three spectral regions: (a) 6.0–7.0 ppm containing the aromatic resonances (tyrosine and histidine),

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