lac Repressor: A Proton Magnetic Resonance Look at the Deoxyribonucleic Acid Binding Fragment†

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ABSTRACT: The DNA binding fragment from *Escherichia coli* *lac* repressor, the N-terminal 56 amino acid residue "headpiece", has been examined by high-resolution ^{1}H NMR spectroscopy at 360 MHz. The aromatic region has been examined in detail along with the four headpieces of altered repressors that are each missing one of the tyrosines, respectively. The spectra here show more resolved resonances and correct errors in the resonance assignments that have been published by Ribeiro et al. (1981b) [Ribeiro, A. A., Wemmer, D., Bray, R. P., Wade-Jardetzky, N. G., & Jardetzky, O. (1981) *Biochemistry 20*, 818–823]. These corrections allow an interpretation of the spectroscopic observations that is now consistent with the extensive genetic analysis that has been carried out with the *lac* repressor gene. In addition, nuclear Overhauser enhancement measurements give a guide to the interresidue distances among the aromatic residues in this protein fragment.

We are interested in the nature of the interaction between the proteins that regulate gene expression and their site of action on the DNA. The *lac* operon has historically been the model system for such studies. The product of the *lac I* gene, the *lac* repressor, regulates the *lac Z* gene expression by preventing RNA polymerase from making *lac Z* mRNA. This occurs by a tight interaction between the repressor and the *lac* operator (Jacob and Monod, 1961). Since the entire primary sequence of the DNA coding for the *lac I* gene product (repressor) along with its operator binding site and the sequence of the *lac* repressor protein have been determined for *Escherichia coli* (Farabaugh, 1978; Beyreuther, 1978), the *lac* operon system continues to be ideal prototype system for studying how gene regulation occurs (Bourgeois & Pfahl, 1976; Miller & Reznikoff, 1980). In wild-type cells, the *lac* operon repressor is a tetrameric protein of 360 amino acids per subunit, without either bound metal ions or cofactors (Bourgeois & Pfahl, 1976), with a total molecular weight of 154 520. We have undertaken an extensive genetic analysis of the relationship between the primary structure of the *lac* repressor and its function (Miller et al., 1979). This paper describes a continuation of our investigation of *lac* repressor structure.

We describe here the ^{1}H NMR spectrum and nuclear Overhauser enhancement (NOE) measurements of a DNA binding peptide derived from the *lac* repressor which retains both specific (Ogata & Gilbert, 1978, 1979) and nonspecific DNA binding properties (Jovin et al., 1977). This peptide represents the N-terminal 56 amino acids (molecular weight of 6225) of the *lac* repressor subunit derived by limited chymotrypsin digestion of the intact tetramer. It is one of a series of peptides containing the first 50–60 amino acids that can be derived by proteases or genetic means (Beyreuther, 1978; Geisler & Weber, 1977; Weber & Geisler, 1980) which shows DNA binding. In the case of limited proteolytic digestion of the *lac* repressor, one derives, as described by Platt et al. (1973), four N-terminal fragments ("headpieces") and a tetrameric core. The core contains the binding sites for the low molecular weight inducers and antiinducers of the *lac* operon as well as part of the *lac* operator DNA binding site (O'Gorman et al., 1980; Matthews, 1979). Chymotrypsin generates a homogeneous headpiece peptide of 56 amino acid residues (Geisler & Weber, 1978) in contrast to trypsin which yields a mixture of peptides 51 and 59 amino acids in length (Platt et al., 1973).

In addition, we show here that the extensive genetics that we have carried out in the *lac I* gene can be used for the interpretation of the aromatic region of the ^{1}H NMR spectrum. Here, we extend the techniques that we have used in the complete assignment of the ^{19}F NMR resonances in the spectrum of 3-fluorotyrosine-substituted *lac* repressor tetramers (Jarema et al., 1981). Our approach to resonance assignments in the NMR spectrum is to systematically alter one, and only one, amino acid residue at a time by genetic means. For the *lac I* gene, we have generated 90 nonsense mutations, which include all eight tyrosine codons for the protein (Miller et al., 1979). By isolating the *lac* repressor from a series of nonsense suppressor strains of *E. coli* with a chromosome containing one of the nonsense mutations in the *lac I* gene, it is possible to substitute either serine, glutamine, leucine, or lysine as well as to reinsert tyrosine at each of the tyrosine positions in the polypeptide chain (Gorini, 1970; Garen, 1968). The specific substitution of leucine for tyrosine, Su6 as we use here, for example, results in a *lac* repressor protein giving a very specific alteration in the aromatic part of the ^{1}H NMR spectrum. By comparison of a complete set of these spectra from altered *lac* repressor headpieces, it is possible to correlate spectral features to specific residues in the amino acid sequence of the normal *lac* repressor headpiece.

The headpiece that we are examining normally contains four tyrosines (residues 7, 12, 17, and 47), one histidine (residue 29), and neither phenylalanines nor tryptophans. Here, we have selectively substituted a leucine for each of the four tyrosines by nonsense suppression. The selective deuteration of either the 3,5 or the 2,6 protons in the tyrosines, in addition to the genetically altered headpieces, allows us to derive complete assignment information for the aromatic region of the NMR spectrum of the *lac* repressor headpiece.

Having assigned the aromatic resonances in the ^{1}H NMR spectrum, we have used a series of nuclear Overhauser enhancement (NOE) measurements to explore the structure of

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the *lac* repressor headpiece. These measurements were made on the selectively deuterated headpieces which eliminates contributions from intratyrosine coupling interactions.

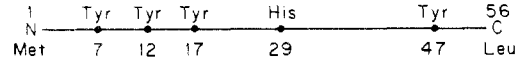
Experimental Procedures

Source of lac Repressors. Wild-type *lac* repressor was obtained by temperature induction of CSH 46 (Miller, 1972). Altered *lac* repressors were obtained by heat induction of *E. coli* strains constructed as follows: *lac* repressor *lac I* gene amber mutations were recombined onto λ phage with a temperature-sensitive cI repressor in strains with an Arg E^- amber mutation, then transduced to Arg^+ with lysates from an Arg E^-Su6^+ strain, and scored for the presence of an i^+ phenotype. The wild-type and altered *lac* repressors were purified as described previously (Lu et al., 1976).

Isolation of the N-Terminal Headpiece. We found that the published procedures (Geisler & Weber, 1978) for the isolation of the *lac* repressor headpiece were not adequate for obtaining the peptide in sufficient quantities for the NMR measurements. In addition, ammonium sulfate precipitation, which led to the denaturation of significant amounts of the peptide, limited the concentration of the headpiece for NMR due to incomplete precipitation and osmotic dilution during dialysis. Thus, the following procedure was used:

The N-terminal headpiece of the *lac* repressor was obtained by digestion of the *lac* repressor (10–50 mg/mL) with 3% (w/w) chymotrypsin (Worthington) in 1 M Tris-HCl (pH 7.5), 30% glycerol, and 10^{-4} M dithiothreitol at 25 °C. After 3 h, digestion was stopped by cooling to 4 °C and adding phenylmethanesulfonyl fluoride (PMSF) to 2 mg/mL. The solution, 10–20 mL, was twice dialyzed for 3 h against 4 L of buffer A [0.06 M phosphate, pH 7.4, 5% glycerol, 10^{-4} M ethylenediaminetetraacetic acid (EDTA), 10^{-2} M 2-mercaptoethanol, and 2.5×10^{-4} M PMSF] before being loaded onto a 2-mL phosphocellulose column. The column was rinsed with 50 mL of buffer A and eluted with a 0.42 M phosphate (5% glycerol) step gradient in a volume of about 3 mL. The 3 mL was loaded onto a 2 × 60 cm Sephadex G-75 (Pharmacia) column equilibrated with buffer A. The headpiece fractions were pooled and loaded onto a 0.7-mL phosphocellulose column in a 1-mL disposable plastic syringe, rinsed with 10 mL of 0.06 M phosphate (no glycerol) buffer, and eluted in about 0.4 mL with 0.42 M phosphate (no glycerol) buffer. The peak fractions, about 0.4 mL, were dialyzed against an equal volume of NMR buffer (0.2 M NaCl, 0.012 M phosphate, and 2×10^{-5} M EDTA in 99.8% D_2O) in a dialysis cell. At least 15 changes of NMR buffer were used followed by 100% D_2O NMR buffer for the last 5 changes. In all cases, the isolated headpiece had fluorescence spectral characteristics of tyrosine with λ_{max} excitation of 275 nm, indicating no contamination by tryptophan-containing proteins (i.e., core or chymotrypsin). Concentrations were measured by using $A_{280} = 0.77$ for the wild-type headpiece and $A_{280} = 0.58$ for the altered headpiece, both at 1 mg/mL.

The headpieces that are isolated here from the wild-type repressor have the following structure:



These headpieces are active in DNA binding by three criteria: (1) We see specific UV difference spectra when calf thymus DNA and *lac* operator DNA fragments are titrated with the headpiece. (2) These headpieces stick to phosphocellulose at 0.2 M phosphate while denatured headpieces do not (Schlotmann & Beyreuther, 1979). (3) We observe specific salt-dependent changes in the NMR spectra of headpiece–DNA fragment complexes (Nick et al., 1981).

Deuterated tyrosine was incorporated into the *lac* repressor by the same procedure we used for 3-fluorotyrosine incorporation (Lu et al., 1976). Tyrosine-*3,5-*d_2 was prepared by refluxing L-tyrosine in 6 N D_2SO_4 in D_2O (Matthews et al., 1977) with three changes of D_2O. Tyrosine-*2,6-*d_2 was prepared by refluxing tyrosine-*2,3,4,5-*d_4 in 6 N H_2SO_4 in H_2O with two changes of H_2O. Tyrosine-*2,3,4,5-*d_4 was obtained by feeding perdeuteriophenol to *Erwinia herbicola*, which have β-tyrosinase activity (Enei et al., 1973). The isotopic purity of the deuterated tyrosines was determined by ^1H NMR before incorporation into the protein. The deuterium substitution was 99% for the tyrosine-*3,5-*d_2 and 95% for the tyrosine-*2,6-*d_2. The isotopic purity of the tyrosine-*2,6-*d_2 was limited by the isotopic purity of the phenol-d_5 (>95% deuterium substitution) that was fed to *Erwinia herbicola*.

NMR spectra were recorded on a WH-360 Bruker spectrometer equipped with a 5-mm probe using 8K data points, a 5000-Hz sweep width, and an acquisition time of 0.8 s. The chemical shifts are reported relative to an external reference of 4,4-dimethyl-4-silapentanesulfonate (DSS) in NMR buffer. Headpiece concentrations ranged from 1×10^{-3} to 5×10^{-3} M in about 0.4 mL. All pH values are meter readings at the reported temperature. All nuclear Overhauser difference spectra were performed by alternately collecting ten scans off resonance and then ten scans on resonance, until the required number of scans were accumulated. For the steady-state Overhauser effects, the resonance was preirradiated for 2 s and the free induction decay accumulated without decoupler irradiation, followed by a recycle delay of 0.2 s. Transient Overhauser effects were observed by inverting the selected resonance with a 20-ms pulse, followed by a variable delay, τ, prior to collecting the free induction decay. A recycle delay of 2 s was used.

Results

The ^1H NMR spectrum at 360 MHz of the *lac* repressor headpiece is shown in Figure 1A. The sine bell resolution enhanced spectrum (De Marco & Wüthrich, 1976) with base-line correction is also shown for the sake of clarity (Figure 1B). From a pH titration, the identification of the C-2 and C-4 resonance from the single histidine residue at position 29 is straightforward. In the subsequent figures, the C-2 histidine resonance has been truncated off. Since there are no phenylalanines or tryptophans in this peptide, the remaining resonances all arise from the four tyrosines at positions 7, 12, 17, and 47. Assuming that all of the tyrosine phenolic groups flip fast with respect to the chemical shift, one would anticipate four pairs of coupled doublets in the spectrum shown in Figure 1A. The identification of these coupled doublets was determined by time-sharing homonuclear decoupling, and they are labeled with Roman numerals I–IV in Figure 1B.

The data in Table I show that we have accounted for all of the 18 protons in the aromatic portion of the NMR spectrum where the areas under the groups of resonances labeled α–δ are tabulated. The upfield group of peaks, δ, gives slightly lower than the anticipated area because this group contains the resonance with the longest T_1 value and our delay time of 2 s is not long enough. In any case, the sine bell resolution enhanced spectrum clearly shows the expected number of resolved resonances, with reduced intensity.

Figure 2 shows the identification of each doublet in the wild-type ^1H NMR spectrum as either 3,5 protons or 2,6 protons (see inset of Figure 1B). The upper and lower spectra in this figure were taken with headpieces isolated from bacteria containing the wild-type *lac I* gene grown in the presence of

Table I

	His-29-C-2	α	His-29-C-4	β	γ	δ
area[a]	1[a]	3.98	1.00	3.94	1.63	4.70
expected area	1	4	1	4	2	6
app T_1 (s)	1.3	1.2	1.3	1.3[b]/1.1[c]	1.1	1.7[b]/1.4[c]
Ribeiro et al. (1981b) nomenclature		$A_L + B_L$		$A_u + C_L$	C_u	$B_u + D_L + D_u$

[a] Area normalized to the downfield His-29 C-2 resonance. [b] Downfield doublets of the group, i.e., I*m* and IV*o*. [c] Upfield doublets of the group, i.e., III*o* and IV*m*.

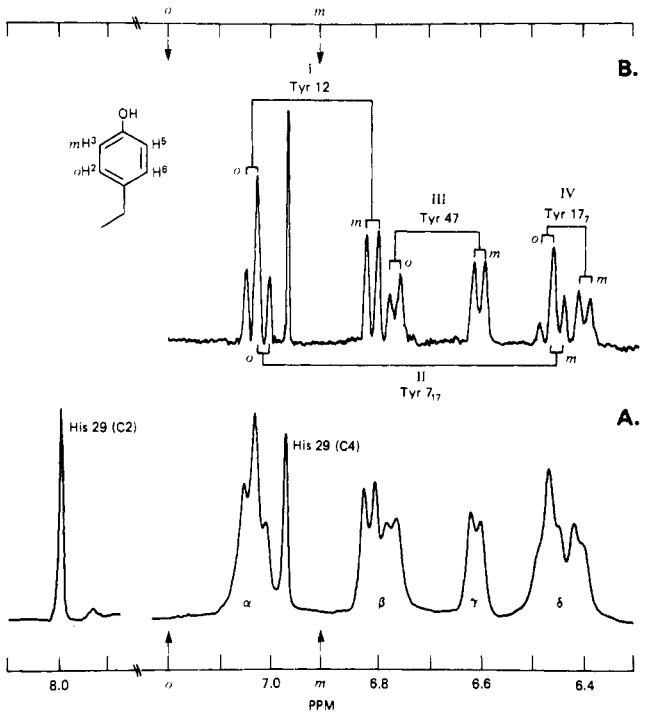


FIGURE 1: ^{1}H NMR 360-MHz spectra of 5 mM wild-type headpiece at 25 °C in 0.2 M NaCl and 0.012 M potassium phosphate buffer, pH 7.1, in D_2O. (A) Block averaged with 30 blocks of 1000 scans each, no line broadening. The areas of the resonances indicated by α–δ are given in Table I. (B) Sine bell apodizatin of the spectrum of (A) with base-line correction. The 2,6 protons are labeled with *o*, and the 3,5 protons are labeled with *m*. The arrows on the axis labeled *o* and *m* give the positions of free tyrosine 2,6 and 3,5 protons, respectively, in the above buffer.

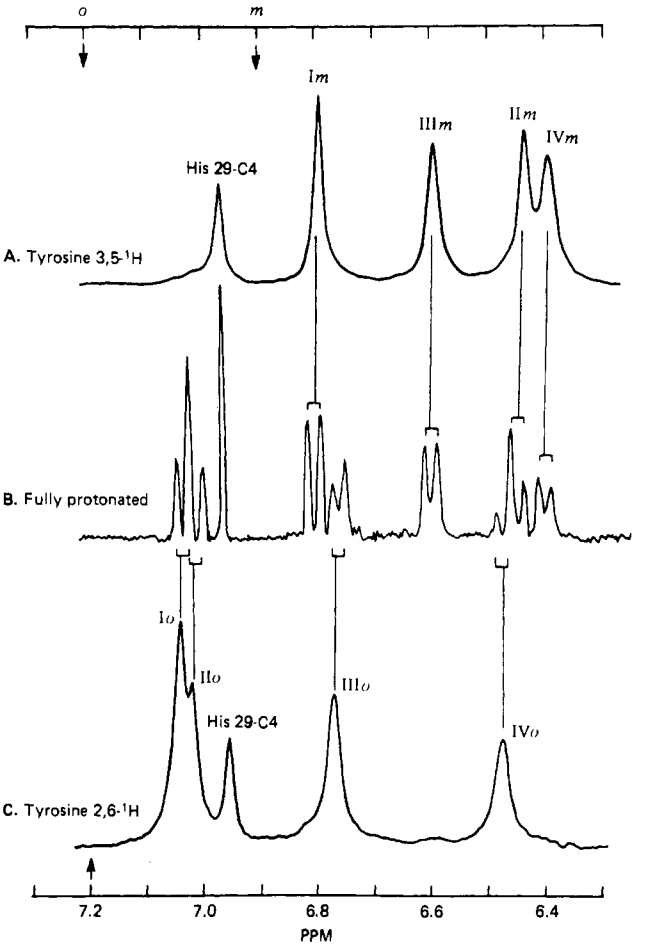


FIGURE 2: (A) Spectrum of 4 mM tyrosine 3,5-^{1}H headpiece. Conditions were as follows: 5 blocks of 1000 scans each, 25 °C, pH 7.1, NMR buffer (see text), no line broadening. The position of the arrow on the upper axis is for free tyrosine 3,5 protons at these conditions. (B) Wild-type sine bell spectrum, same conditions as in Figure 1B. (C) Spectrum of 4 mM tyrosine 2,6-^{1}H headpiece. Conditions were as follows: 5 blocks of 1000 scans each, 25 °C, pH 7.15, NMR buffer, no line broadening. The position of the arrow on the lower axis is for free tyrosine 2,6 protons at these conditions.

the appropriate deuterated tyrosine. The spectra show a single peak for each tyrosine, centered at their respective doublets. At this point, we are able to establish which pairs of doublets are from the same tyrosine and whether the doublet of each pair is tyrosine 3,5 (meta) or 2,6 (ortho) protons. This is indicated both in Figure 1B and by Roman numerals with *o* or *m*.

Assignment of the Resonances in the NMR Spectrum. The strategy that we have taken for the assignment of the resonances involves the isolation of the headpiece from four altered *lac* repressors where, one at a time, each of the tyrosines has been substituted with leucine and the examination of their respective NMR spectra. As one might expect, some tyrosines are more important than others for *lac* repressor function or structure. It has been possible to look at the properties of the intact repressor when specific amino acids have been substituted by nonsense suppression at each of the tyrosine positions in turn. This information, for the four N-terminal tyrosines of interest here, which is a small portion of the genetic information that we have previously published (Miller et al., 1979), is summarized in Table II. Note that these data are for the intact tetrameric repressor. From the results of the work here, we can add to this table the fact that the substitution of tyrosine with leucine does not alter the structure of

Table II: Effect of Amino Acid Substitution at the Four N-Terminal Tyrosine Positions[a]

wild-type amino acid position	Ser	Gly	Tyr	Leu	Lys
Tyr-7	+[b]	+	+	+	+
Tyr-12	+	+	+	+	+
Tyr-17	−[c]	−	+	∓	−
Tyr-47	−	−	+	−	−

[a] From Miller et al. (1979). [b] Normal operator DNA binding. [c] No operator activity DNA binding.

the intact repressor sufficiently to change the results of the chymotrypsin digestion. The headpiece is obtained in identical yields under identical conditions as for wild-type repressor.

Leucine was chosen to substitute for the tyrosines because it gave intact repressors with at least partial wild-type operator

Table III: Areas in Figure 3

spectrum	resonance no. 1	2	3	4	5	6
(B) Tyr-47 → Leu	4.7 (4)[a]	0.91 (1)	1.8 (2)	5.3 (6)		
(C) Tyr-12 → Leu	2.0 (2)	0.94 (1)	2.4 (2)	2.0 (2)	2.0 (2)	4.2 (4)
(D) Tyr-17 → Leu	1.8 (2)	2.0 (2)	0.97 (1)	4.3 (4)	2.0 (2)	2.0 (2)
(E) Tyr-7 → Leu	~2 (2)	0.9 (1)	~8 (8)	1.8 (2)		

[a] Expected values are given in parentheses. Area normalized to histidine-29 (C-2).

Table IV: Inferences from Figure 3

altered headpiece spectrum				
Tyr-47 → Leu	I = $s \neq$ Tyr-47		III = Tyr-47	
Tyr-12 → Leu	I = Tyr-12	II = $u \neq$ Tyr-12	III = $v \neq$ Tyr-12	
Tyr-17 → Leu	I = $y \neq$ Tyr-17	II $\cong x \neq 17$; $x = 7$	III = $z \neq$ Tyr-17	IV $\cong$ Tyr-17
Tyr-7 → Leu		II = Tyr-7/17		IV = Tyr-7/17
consensus:	I = Tyr-12	II $\cong$ Tyr-7	III = Tyr-47	IV $\cong$ Tyr-17
Ribeiro et al. (1981b) nomenclature:	A	B	C	D

DNA binding activity in three of the four tyrosine positions. The expected result for each of the altered repressors is a ^{1}H NMR spectrum missing a pair of doublets. The simultaneous removal of four resonances (doublet pair) with a change in the structure of the peptide complicates the interpretation of the NMR spectra from the altered repressors. Thus, the assignment that we propose here depends on an interpretation that is consistent with *all* of the altered spectra. Absolute assignment of any one tyrosine doublet pair by looking at just one spectrum for a specific altered repressor is not prudent. With this in mind, consider the collection of altered *lac* repressor headpiece NMR spectra in comparison with the wild-type spectrum as shown in Figure 3. The coupled doublets indicated by the lines labeled *s* and *u–z* were determined by time-sharing homonuclear decoupling. To avoid confusion, we will refer to all of the coupled doublets in the wild-type spectrum by Roman numerals I–IV and the features in the spectra of the altered headpieces by lower case letters *s–z*. The integrated areas for these spectra are found in Table III.

Comparison of the spectrum from the headpiece where tyrosine-47 has been substituted with a leucine (Figure 3B) with the wild-type spectrum (Figure 3A) suggests that the set of doublets labeled III arises from tyrosine-47 since it is the feature that is missing. Doublet pair I is *s*, II*o* is the third component in the downfield portion of *s*, and II*m* is most likely in *t*. Set IV is then buried in *t* as well. The entire spectrum of the headpiece with tyrosine-47 substituted with leucine can be explained by the disappearance of doublet pair III (tyrosine-47) and a broadening of the upfield resonances.

A comparison of the NMR spectrum from the headpiece where tyrosine-12 has been substituted with leucine (Figure 3C) with the wild-type spectrum suggests that doublet pair I comes from tyrosine-12 since it is the feature that is missing. The remainder of the spectrum is basically unchanged. We account for all of the remaining resonances by noting that set II is *u*, set III is *v*, and set IV is *w*.

The NMR spectrum of the headpiece where tyrosine-17 has been substituted with leucine reflects one of the problems common to all approaches involving chemically modified or variant proteins—several simultaneous alterations in the NMR spectrum due to a single change in primary structure. There are clearly resonances missing from the upfield end of the spectrum, but significant shifts of other resonances are also occurring in the remainder of the spectrum. Given the coupling pattern indicated in the figure, we would account for doublet pair I by *y* and doublet pair III by *z*. This forces one to suggest that *x* arises from tyrosine-7, but we cannot be sure whether it was originally doublet pair II or pair IV. It was hoped that an examination of the NMR spectrum from the headpiece where tyrosine-7 has been substituted with leucine would settle the uncertainty. As one can see from the spectrum (Figure 3E), it does not help. In fact, all of the resonances are quite broad, and it was not possible to determine the coupling pattern by homonuclear decoupling. Note, however, the upfield resonances at 6.4–6.5 ppm are missing. We are thus left with the conclusion that doublet pair II and pair IV are tyrosine-7 and tyrosine-17, respectively. As we indicate in Figures 1B and the top of Figure 3, we suggest that doublet II is tyrosine-7 since it has the larger downfield shift in its 2,6 protons (i.e., resonance II*o*, Figure 1B or 3). In the subsequent discussion, we will refer to doublet pair II as tyrosine-7/17 and doublet pair IV as tyrosine-17/7, keeping in mind that some ambiguity in the assignment of these two tyrosines remains.

Table IV presents a summary of the inferences leading to the resonance assignments presented here.

Nuclear Overhauser Effects. In order to derive information about the spatial distribution of the tyrosines in the *lac* repressor headpiece, we have performed a systematic series of nuclear Overhauser enhancement measurements. These NOE measurements have been done with the specifically deuterated forms of the peptide to simplify the analysis of the data.

The advantages of using the selectively deuterated *lac* headpiece are 2-fold. First, the 2,6 or 3,5 tyrosine deuteration reduces the number of tyrosine resonances by a factor of two, which allows one to more easily select irradiation resonances in otherwise overlapping resonances. Second, selective deuteration eliminates intratyrosine coupling and Overhauser effects, eliminating relaxation of the 3,5 protons by the 2,6 protons and vice versa. The steady-state Overhauser effects are shown in Figure 4 for the tyrosine 3,5-^{1}H headpiece (i.e., deuterated on the 2,6 position) at 4 °C. Plotted as difference spectra, negative Overhauser effects appear as positive peaks. In this figure, the resonance marked with the arrow was irradiated prior to the accumulation of the free induction decay. Table V presents the calculated steady-state nuclear Overhauser effects, η, using the expression (Noggle & Schirmer, 1971)

$$\eta = \frac{I - I_o}{I_o}$$

where I is the intensity of the observed resonance when a spin is saturated and I_o is the intensity of the observed resonance

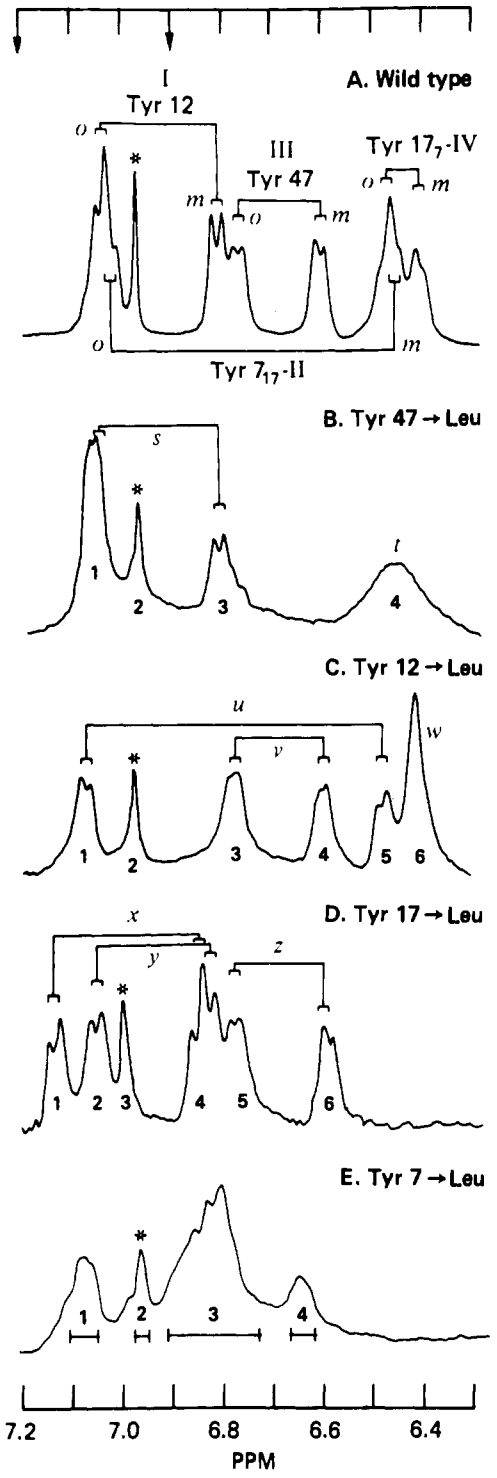


FIGURE 3: All spectra recorded at 25 °C. (A) Wild-type headpiece spectrum, same conditions as in Figure 1A. (B) Spectrum of headpiece where tyrosine-47 is replaced by leucine (1 mM) at pH 7.1, 5 blocks of 1000 scans each. (C) Spectrum of headpiece where tyrosine-12 is replaced by leucine (1.5 mM) at pH 7.1, 5 blocks of 1000 scans each. (D) Spectrum of headpiece where tyrosine-17 is replaced by leucine (1 mM) at pH 7.0, 5 blocks of 1000 scans each. (E) Spectrum of headpiece where tyrosine-7 is replaced by leucine (1 mM) at pH 7.2, 5 blocks of 1000 scans each. All spectra have 1-Hz line broadening and are plotted so the areas of the histidine-29 C-2 proton (at about 8 ppm) are equal. The histidine-29 C-4 proton is labeled with the asterisk. Areas, determined by cutting and weighing and normalized to the histidine-29 C-2 proton, are given in Table III.

without irradiation of the other spin. Under the conditions of these experiments, the decoupler irradiation directly changes the intensity of resonances that are close together. This problem occurs with resonances II*m* and IV*m* which are only

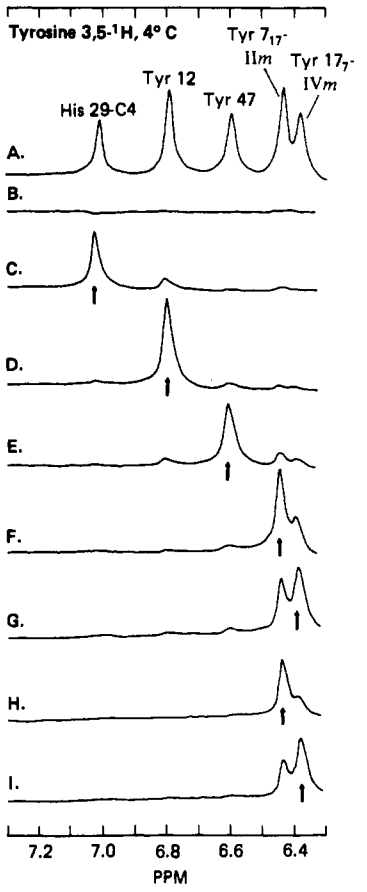


FIGURE 4: Steady-state Overhauser spectra of tyrosine 3,5-^{1}H headpiece, 4 mM, at 4 °C. All spectra have 1-Hz line broadening. (A) Spectrum of 3,5-^{1}H headpiece, 200 scans. (B–I) Difference spectra of the spectrum accumulated with 200 scans of on-resonance irradiation (at the frequency marked by the arrow) minus the spectrum accumulated with 200 scans of off-resonance irradiation. (B) His-29 C-2 proton at 8.00 ppm irradiated. (C) His-29 C-4 proton irradiated. (D) Tyrosine-12 3,5-^{1}H irradiated. (E) Tyrosine-47 3,5-^{1}H irradiated. (F) Tyrosine-7/17 3,5-^{1}H (resonance II*m*) irradiated. (G) Tyrosine-17/7 3,5-^{1}H (resonance IV*m*) irradiated. (H) Selective irradiation of resonance II*m*. (I) Selective irradiation of resonance IV*m*. All spectra have same *Y* expansion. The NOE values are given in Table V.

Table V: NOE Values, 4 °C

	residue observed				
residue irradiated	His-29 C-4	Tyr-12	Tyr-47	Tyr-7/17 (II*m*)	Tyr-17/7 (IV*m*)
His C-2	0	0	0	−0.02	−0.03
His C-4	*x*	−0.12	0	−0.04	0
Tyr-12	−0.07	*x*	−0.10	−0.05	−0.04
Tyr-47	−0.03	−0.09	*x*	−0.14	−0.12
Tyr-7/17 (II*m*)	−0.04	−0.03	−0.10	*x*	−0.57[b]
Tyr-17/7 (IV*m*)	−0.04	−0.03	−0.09	−0.61[b]	*x*
Tyr-7/17 (II*m*)[a]	−0.04	0	−0.04	*x*	−0.30
Tyr-17/7 (IV*m*)[a]	−0.03	−0.03	−0.05	−0.40	*x*

[a] Selective irradiation. [b] Overestimate; see text.

0.055 ppm apart. Thus, the measured Overhauser effects for peaks II*m* and IV*m* (tyrosine-7 and -17) in Figure 4 spectra F and G are too large. The decoupler power was reduced so that irradiation at 0.055 ppm upfield from resonance IV*m* caused no change in its intensity. The NOE measurements were then repeated for peaks II*m* and IV*m*. Since the irradiation power was reduced to a minimum, the values for selective irradiation of tyrosines-7 and -17 are a lower limit for the NOE values between them. A comparison of the NOE

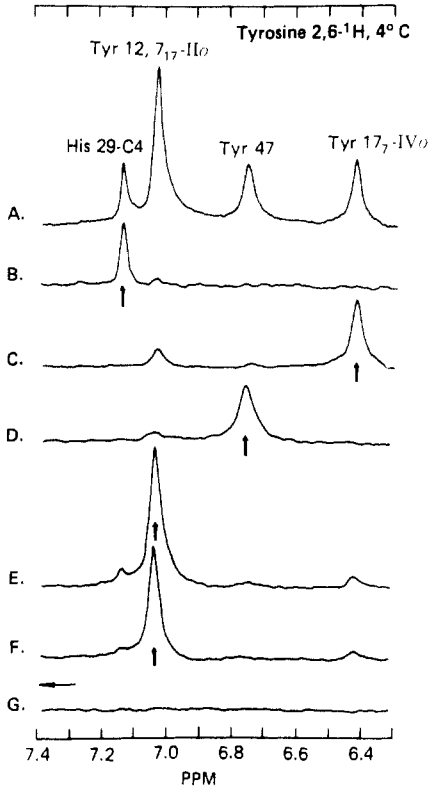


FIGURE 5: Steady-state Overhauser spectra of tyrosine 2,6-^{1}H headpiece, 4 mM, at 4 °C. All spectra have 1-Hz line broadening. (A) Spectrum of 2,6-^{1}H headpiece, 200 scans. (B–G) Difference spectra of the spectrum accumulated with 200 scans of on-resonance irradiation (at the frequency marked by the arrow) minus the spectrum accumulated with 200 scans of off-resonance irradiation. (B) His-29 C-4 proton irradiated. (C) Tyrosine-17/7 3,5-^{1}H (resonance IV*m*) irradiated. (D) Tyrosine-47 3,5-^{1}H irradiated. (E) Tyrosine-12 and tyrosine-7/17 (resonance II*o*) 3,5-^{1}H irradiated. (F) Selective irradiation of tyrosine-12 and tyrosine-7/17 (resonance II*o*) 3,5-^{1}H. (G) Irradiation of histidine-29 C-2 proton at 8.2 ppm. All spectra have the same *Y* expansion. The NOE values are given in Table VI.

Table VI: NOE Values, 4 °C

	residue observed			
residue irradiated	His C-4	Tyr-12 + Tyr-7/17 (II*o*)	Tyr-47	Tyr-17/7 (IV*o*)
His C-2	0	0	0	0
His C-4	*x*	−0.004 to −0.008	0	0
Tyr-12 + 7/17 (II*o*)	−0.18	*x*	−0.09	−0.18
Tyr-12 + 7/17 (II*o*)[a]	−0.10	*x*	−0.05	−0.14
Tyr-47	0	−0.05 to −0.11	*x*	0
Tyr-17/7 (IV*o*)	0	−0.11 to −0.22	−0.07	*x*

[a] Selective irradiation.

values indicates that the 3,5 protons of tyrosine-7 are very close to the 3,5 protons of tyrosine-17. (Note that the 5% 2,6 ^{1}H in tyrosine-*2,6-d*$_2$ would lead to a maximum NOE error of +0.025.)

A similar series of NOE measurements were made on the headpiece containing tyrosines with its ring protons only at the 2,6 positions. The spectra are in Figure 5, and the corresponding NOE values are in Table VI. Since the resonance due to the His-29 (C-4) proton and the resonance due to both tyrosine-12 and tyrosine-7/17 (resonance II*o*) are quite close, the irradiation power was reduced for more selectivity. Reduced irradiation at the His-29 (C-4) resonance resulted in no changes in the NOE values while reduced irradiation at the combined tyrosine-12 + tyrosine-7/17 resonance did give slightly altered NOE values.

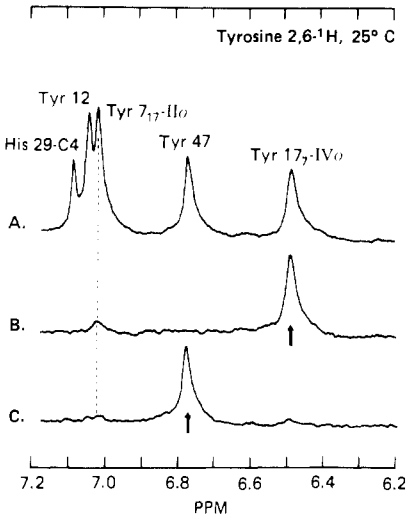


FIGURE 6: Steady-state Overhauser spectra of tyrosine 2,6-^{1}H headpiece, 4 mM, at 25 °C. All spectra have 0.5-Hz line broadening. (A) Spectrum of the 2,6-^{1}H headpiece, 200 scans. (B and C) Difference spectra as described in Figure 5 accumulated with 200 scans on resonance minus 200 scans off resonance. (B) Tyrosine-17/7 (resonance IV*o*) 2,6-^{1}H irradiated. (C) Tyrosine-47 2,6-^{1}H irradiated. All spectra have the same *Y* expansion. The NOE values are given in Table VII.

Table VII: NOE Values, 25 °C

	residue observed			
residue irradiated	Tyr-12	Tyr-7/17 (II*o*)	Tyr-47	Tyr-17/7 (IV*o*)
III*o*	−0.03	−0.06	*x*	−0.06
IV*o*	0	−0.16	0	*x*

At 4 °C, the temperature used for the series of NOE measurements described above, we have the problem of the superimposition of the resonances from tyrosine-12 and tyrosine-7/17 (resonance II*o*) 2,6 protons as indicated by the resonance at 7.02 ppm in Figure 5A. The NOE experiment was repeated at 25 °C to identify which tyrosine changes intensity (Figure 6). When peak IV*o* was irradiated, only peak II*o* changed intensity with an Overhauser effect of −0.13. As we noted above, resonances II*o* and IV*o* correspond to tyrosine-7 and tyrosine-17. The Overhauser effects at 25 °C (Table VII) are less than those at 4 °C due to a change in the rotational correlation time of the protein. If a conformational change that changes the relative positions of the tyrosines between 4 and 25 °C does not occur, then the Overhauser effect at 4 °C of the resonance due to tyrosine-12 and II*o* can be attributed entirely to peak II*o*, with an Overhauser effect of −0.22. Since irradiation of the resonance at 7.05 ppm causes an Overhauser effect of −0.18 for peak IV*o* (Figure 5E), we conclude, as with the 3,5 protons, that the Overhauser effects show the tyrosine-7 2,6 protons are very close to the tyrosine-17 2,6 protons. This statement does not require the absolute assignment of doublet pair II to tyrosine-7 and pair IV to tyrosine-17 or vice versa.

When making steady-state NOE observations in macromolecules, one must be sure that the correlation time for isotropic molecular motion is sufficiently small. Otherwise, significant diffusion of magnetization among coupled spins occurs, making any conclusion about distance between nuclei less useful (Kalk & Berendsen, 1976; Bothner-By & Noggle, 1979; Hull & Sykes, 1975). In order to assure ourselves that the *lac* repressor headpiece is small enough to allow one to ignore spin diffusion, we performed the transient NOE measurement with the selectively deuterated headpiece containing

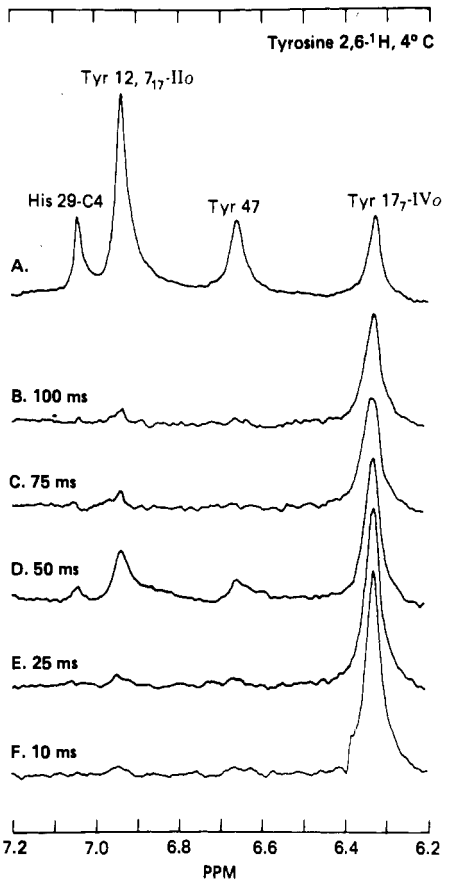


FIGURE 7: Transient Overhauser spectra of tyrosine 2,6-^{1}H headpiece, 4 mM, at 4 °C. All spectra have 1.5-Hz line broadening. (A) Spectrum of tyrosine 2,6-^{1}H headpiece, 200 scans. (B–F) Difference spectra of the spectrum accumulated (200 scans) at 10, 25, 50, 75, or 100 ms after tyrosine-17/7 (resonance IV*o*) is inverted by a 20-ms pulse minus the spectrum accumulated (200 scans) at 10, 25, 50, 75, or 100 ms, respectively, after an off-resonance 20-ms pulse. Spectra B–F have 2× the *Y* expansion of spectrum A.

2,6 tyrosine protons shown in Figure 7. Resonance IV*o* (tyrosine-17 2,6 protons) was chosen for the 20-ms pulse (50-Hz half-width) since it was well separated (120 Hz) from the nearest tyrosine 2,6 proton resonance (tyrosine-47).

In this experiment, the initial buildup rate of the NOE is directly related to the distance of the proton from the IV*o* protons. NOE difference spectra were collected at 10, 25, 50, 75, and 100 ms after the end of the 20-ms pulse. After the pulse, resonance IV*o* decays exponentially, transferring its magnetization via relaxation to other protons at rates characteristic of the cross-relaxation coefficients. In the difference spectra, the resonances of these other protons build to a maximum at about 50 ms and then decay. The resonance of tyrosine-12 and -7/17 (resonance II*o*) and the resonance III*o* both show area at τ = 10 ms, with the histidine C-4 resonance appearing between 25 and 50 ms. Shorter values of τ and higher signal to noise ratios would be needed to differentiate the buildup rates of the combined resonance due to tyrosine-12 and -7/17 from the resonance due to tyrosine-47. The fact that the relative NOE values in the transient experiment are quite similar to the steady-state NOE values allows us to assert that there is very little spin diffusion, if any, occurring here.

Tyrosines-7 and -17 Are Very Close to Each Other and Possibly Stacked Together. From the NOE experiments, it is clear that tyrosine-7 and tyrosine-17 are very close to each other, even though we cannot determine for sure which set of resonances belongs to which of these two tyrosines. Several observations among the spectra shown here suggest that these

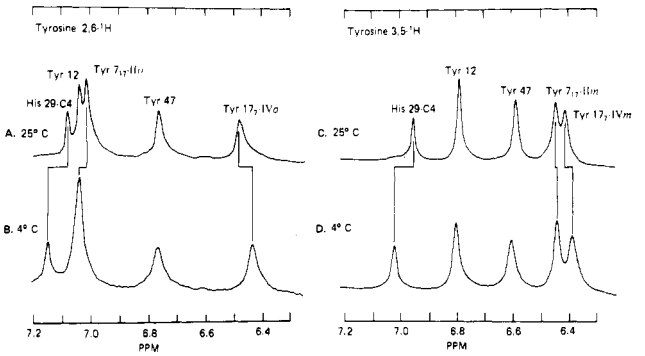


FIGURE 8: Temperature dependence of the aromatic resonances of the headpiece. (A) Tyrosine 2,6-^{1}H headpiece: 4 mM, 25 °C, 500 scans. (B) Tyrosine 2,6-^{1}H headpiece: 4 mM, 4 °C, 500 scans. (C) Tyrosine 3,5-^{1}H headpiece: 4 mM, 25 °C, 500 scans. (D) Tyrosine 3,5-^{1}H headpiece: 4 mM, 4 °C, 500 scans. All spectra have 1-Hz line broadening.

two tyrosines might be ring stacked. The absence of tyrosine-17 causes tyrosine-7 (*x* in Figure 3D) to shift downfield in the headpiece, with tyrosine-17 replaced by leucine, and the absence of tyrosine-7 causes tyrosine-17 to shift downfield in the headpiece with tyrosine-7 replaced by leucine (Figure 2E has no resonances upfield from 6.6 ppm). These data are consistent with tyrosine-7 and tyrosine-17 being ring stacked with mutual upfield shifts. Ring stacking would further explain the large upfield position of doublet IV*o*, a 2,6 proton doublet belonging to one of these tyrosines. However, tyrosine pair IV has both 2,6 and 3,5 protons shifted upfield while tyrosine pair II has only the 3,5 protons shifted upfield. The downfield position of the 2,6 protons of tyrosine pair II could be accounted for by a nonparallel, noncoaxial stacked array of tyrosine-7 and tyrosine-17 and/or by neighboring aromatic rings or charged groups.

For further exploration of this point, the spectra of headpieces with ring protons at the 3,5 or 2,6 positions of the tyrosines at 4 and 25 °C are shown in Figure 8. The notable feature is the downfield shifts of peaks IV*o*, IV*m*, and II*m* with increasing temperature, a result consistent with ring destacking of tyrosines-7 and -17. The chemical shifts of tyrosine-12 and tyrosine-47 resonances are relatively constant between 4 and 30 °C. Histidine-29 undergoes an upfield shift when the temperature is raised from 4 to 25 °C, which is in the opposite direction of the shift expected due to the change in the phosphate buffer pH with increasing temperature. This shift thus reflects a change in the environment of histidine-29 between 4 and 25 °C. Above 30 °C, the headpiece begins to precipitate. Although the argument can be made that selective tyrosine substitution disrupts headpiece structure, we believe that the shifts of the resonances in the tyrosine-12 and tyrosine-47-substituted headpieces are small and that the downfield shifts in the spectrum of Figure 2D (headpiece with tyrosine-17 substituted) are due to ring destacking of tyrosine-7 protons from the missing tyrosine-17 and part of the change in the spectrum in Figure 2E (headpiece with tyrosine-7 substituted) is due to ring destacking of the tyrosine-17 protons from the missing tyrosine-7.

Discussion

Variants of proteins have been used to assign NMR spectral features to amino acid residues, but in the past this has been limited to natural variations between populations or species. This approach has been exploited in staphylococcal nuclease (Roberts & Jardetzky, 1970), hemoglobin (Oldfield & Allerhand, 1975; Fung & Ho, 1975; Weichelman et al., 1976; Viggiano et al., 1978), pancreatic ribonuclease (Migchelsen

& Beintema, 1973; Leijenaar-Van der Berg et al., 1974), and cytochromes (Cohen & Hayes, 1974; Dobson et al., 1975; Oldfield et al., 1975; Keller et al., 1973; Moore & Williams, 1980a,b). What we have done here is to show that it is possible to seek out and use variants in a systematic and comprehensive way to make specific changes at the molecular level for the interpretation of NMR spectra. We first demonstrated this complete genetic approach for the eight resonances in the 3-fluorotyrosine-substituted form of the intact tetrameric *lac* repressor (Jarema et al., 1981).

The data here show that the same strategy can be applied to the more complex ^{1}H NMR spectrum of the *lac* repressor N-terminal fragment where the aromatic region contains 16 resolved resonances representing 18 protons. For the headpiece, the more conventional approach to peak assignment, chemical modification, would encounter difficulties since the reactivities of some of the tyrosine residues toward nitration (Alexander et al., 1977; Hsieh & Matthews, 1981) and iodination (Fanning, 1975) are very similar. This difficulty is clearly illustrated by the incorrect assignments of the ^{1}H resonances using nitration of the tyrosines in the *lac* repressor headpiece which was reported in this journal by Ribeiro et al. (1981b).

The *pairing* of the tyrosine doublets in our work agrees with and confirms that of Ribeiro et al. (1981b).

The assignment strategy of Ribeiro et al. (1981b) uses the correlation of the rate of decrease in resonance intensity with the rate of chemical modification of that residue. The effects of nitrated tyrosine resonances and denaturation on the area determinations of the chemically modified headpiece are less severe for the mild nitration conditions during which tyrosine-7 and tyrosine-17 become modified. Area determinations are thus more accurate for these conditions. We believe that the assignment of tyrosine-7 to doublet pair II (their B) and tyrosine-17 to doublet pair IV (their D) by Ribeiro et al. (1981b) is justified given their data. However, with the problems encountered in the assignments of tyrosines-12 and -47 described below, we await more definitive experiments before absolutely declaring which pair of doublets is tyrosine-7 or -17.

We note that the assignments by Ribeiro et al. (1981b) for tyrosine-12 and tyrosine-47 are in error. Their resonances A should be tyrosine-12, and C should be tyrosine-47 instead of vice versa as they suggest. This confusion is easy to understand since the rates of nitration of these two tyrosines are quite similar. Also, complications arise when one notes that any denaturation as well as nitration that occurs will lead to an increase in area in the region occupied by the resonances from tyrosine-12. Denaturation results in the movement of all of the resonances to the position indicated in Figure 1 of Ribeiro et al. (1981b) for free tyrosine. Nitration of the tyrosine results in a downfield shift of the remaining 3,5 protons. Thus, any rate of loss in the area due to the resonances of tyrosine-12 must be reduced by the rate of increase of both denatured protein and nitrated tyrosines in the protein undergoing modification. With this in mind, an examination of the data in Table II of Ribeiro et al. (1981b) shows that the differences in the loss of area in the tyrosines-12 and -47 resonances are not significant enough to make a positive assignment. In fact, if the upfield resonance of their tyrosine pair A is used to monitor the rate of change of pair A (the lowfield resonance of pair A overlaps with the lowfield resonance of pair B), then tyrosine A loses area faster than tyrosine C [see Figure 2 of Ribeiro et al. (1981b)]. This supports our resonance assignments. These kinds of difficulties have been seen in earlier NMR spectral assignments, for example, the assignment of the four histidines of RNase by pH titration (Markley, 1975).

The advantage of using genetically altered proteins is that one knows the precise position of the amino acid substitution, as well as the fact that there is only one substitution. The locations of the mutation that lead to the altered repressors that we exploit here have been checked by both direct protein and DNA sequence analysis as part of the genetic analyses of the *lac I* gene (Platt et al., 1972; Ganem et al., 1973; Files et al., 1974; Miller et al., 1979). Chemical modification, on the other hand, is always subject either to partial modification or to reactions that modify several positions simultaneously. Either approach suffers from the problem that some amino acid substitutions or chemical modifications lead to larger disturbances in the protein structure with simultaneous multiple changes in the NMR spectrum. Figure 3E is one case where this complication arises.

A qualitative examination of the wild-type spectrum in Figure 1A shows some interesting features. All of the amide protons are exchanged within 6 h at 4 °C (our minimum dialysis time). This observation is consistent with the general notion that the *lac* repressor headpiece is quite mobile (Buck et al., 1978; Wade-Jardetzky et al., 1979; Jarema et al., 1981) and can be contrasted with bovine trypsin inhibitor, where some amide protons require denaturation for exchange (Wagner & Wüthrich, 1979), and bovine pancreatic ribonuclease A, where some of the amide protons require elevated temperatures or many days for exchange (Markley, 1975). In spite of the intrinsic flexibility of the protein, there must be significant structure to yield the range of chemical shifts observed for the four tyrosines. Furthermore, this structure is altered when tyrosine-7 is substituted with leucine. This substitution and the one at tyrosine-17 affect the overall spectrum the most, suggesting that they are important to repressor structure, a result consistent with the genetic data in Table II. One notes also the unusual upfield location of two sets of 2,6 proton doublets. The 2,6 protons of tyrosine-17/7 (peak IV*o* of Figure 1) at 6.48 ppm are located 0.72 ppm upfield from free tyrosine, and the 2,6 protons of tyrosine-47 (peak III*o* of Figure 1) at 6.78 ppm are located 0.42 ppm upfield from free tyrosine.

Chandrasekaran et al. (1979) and Ribeiro et al. (1981b) proposed that tyrosines-7, -12, and -17 exist in a stacked array with a spacing suitable for intercalation into DNA. Ribeiro et al. (1981a) have also proposed a model for the folding of the headpiece based on their, incorrect, tyrosine resonance assignments. In addition, they report that there are no significant intertyrosine NOE's. We do see very significant intertyrosine NOE's because we use selectively deuterated tyrosines. This allows more selective irradiation and overcomes the large effects due to intraring NOE's. Our NOE data and resonance assignments rule out both models. The chemical shift data and the results of our NOE measurements clearly indicate that tyrosine-7 and tyrosine-17 are very close and possibly stacked.

Buck et al. (1980) have published the results of photochemically induced dynamic nuclear polarization experiments exploring the DNA interactions with *lac* repressor headpiece tyrosines. Their interpretation was based on resonance assignments in the ^{1}H NMR spectrum which contained errors in both the pairing of the tyrosine doublets and the misassignment of tyrosines-47 and -12. By use of our assignments, the interpretation of the work of Buck et al. (1980) can be briefly revised as follows: In the free headpiece, tyrosine-47 is less accessible to the photoexcited dye than the tyrosines

at 7, 12, or 17. Upon binding to oligo[d(A-T), access of the dye to tyrosine-7, tyrosine-17, and histidine-29 is blocked, with no change in the inaccessibility of tyrosine-47. Tyrosine-12 remains exposed to the dye in the complex. This is completely consistent with our genetic data which show that tyrosine-12 can be replaced while the presence of the other three N-terminal tyrosines is required for operator DNA binding (Table II).

The exposure of tyrosine-12 to a solvated dye, mentioned above, is in agreement with both the proton and the ^{19}F NMR data (Jarema et al., 1981). With both nuclei, the tyrosine 3,5 positions give resonances nearest that of the respective free tyrosine chemical shifts, reflecting the free solvated nature of the residue. For some reason, this particular residue, although highly solvated by these criteria, is relatively unreactive to nitration (Hsieh & Matthews, 1981).

With the available evidence, it is possible to propose the following model for the headpiece: (a) Tyrosine-12 is at or near the surface of the headpiece with chemical shifts very similar to those for free tyrosine. (b) Tyrosines-7 and -17 are very close, possibly ring stacked, and exist near or at the surface of the protein. (c) Tyrosine-47 is inaccessible in the headpiece and is possibly buried. The NOE data show that tyrosine-47 is closer than tyrosine-12 to tyrosines-7 and -17. Also, the headpiece where tyrosine-47 is replaced with a leucine shows broadening of the upfield resonances of tyrosine-7 and tyrosine-17 (Figure 3B). Tyrosine-47 is thus possibly involved in maintaining some local structure in the headpiece. (d) Histidine-29 is at the surface of the headpiece as seen by its titration behavior and accessibility to the photoexcited dye. The NOE data show that the histidine-29 C-4 proton is closest to tyrosine-12.

We have shown that it is possible to assign NMR resonances in both the [^{19}F]tyrosine-substituted *lac* repressor (Jarema et al., 1981) and the *lac* headpiece by using genetically altered proteins. These assignments yield a consistent picture of the aromatic residues of the *lac* headpiece that agrees with the genetic evidence. Also, the nuclear Overhauser data from the selectively deuterated tyrosine headpieces can be used as a guide for distances between the aromatic resonances of the *lac* headpiece. A program to collect two-dimensional NOE data is in progress and will yield absolute geometrical information from NMR data alone.

Acknowledgments

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Proton Nuclear Magnetic Resonance Study on Uridine Imido Proton Exchange†

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ABSTRACT: The exchange of the N(3)-H imido proton of uridine has been studied in aqueous solution by ^{1}H NMR as a function of pH and temperature. At temperatures ranging from −1 to +30 °C, the minimum exchange rate is found at pH 3.4-4.2. The exchange rate as a function of pH has been interpreted in terms of general base-acid catalysis. The proton-catalyzed exchange rates, $k_{H_3O^+}$, are in the order of 10^4 M^{-1} s^{-1} but the base-catalyzed exchange rates, k_{OH^-}, are in the order of 10^{10} M^{-1} s^{-1}, typical for diffusion-limited processes. The thermodynamic activation parameters from pH 1.7 to pH 5.3 have been evaluated from the temperature dependence of the exchange rate to be $\Delta H^{\ddagger\circ}$ = 7.8 ± 0.8 kcal M^{-1}, $\Delta S^{\ddagger\circ}$ = −22.6 ± 1.8 eu, and $\Delta G^{\ddagger\circ}$ = 14.5 ± 0.6 kcal M^{-1}. The existence of a different predominant exchange mechanism at below pH 5.3 is indicated by a decrease of the activation enthalpy ($\Delta H^{\ddagger\circ} \simeq$ 4.0 kcal M^{-1}) accompanied by a drop of the entropy ($\Delta S^{\ddagger\circ} \simeq$ −32.5 eu); the activation free enthalpy $\Delta G^{\ddagger\circ}$ is not significantly affected by the change of the exchange mechanism. The catalytic effect on the NH exchange rate at pH_{min} (3.7 ± 0.1) has been studied as a function of concentration of the catalysts, phosphate and trifluoroethylamine (TFEA). At 20 °C, the enhancement of the exchange rate by the basic form of phosphate and TFEA is characterized by k_{cat} = 1738 and 2.45 × 10^4 M^{-1} s^{-1}, respectively, when k_{cat}'s are derived from the total concentration of the catalyst, or, taking into account the actual concentration of the basic form of phosphate and TFEA at pH 3.7-3.8, k_{cat} = 7.0 × 10^4 and 2 × 10^6 M^{-1} s^{-1}, respectively. These values are in excellent agreement with the calculated rates according to Eigen's theory of proton transfer processes. Therefore, the phosphate and TFEA can be considered two catalysts with their relative catalytic strength proportionally relative to their respective proton acceptor strength.

The exchange rate of the NH, NH_2, and OH protons of nucleic acids has been widely studied to obtain structural and conformational information (Printz & Von Hippel, 1965; Englander & Englander, 1965; Englander, S. W., et al., 1972; Englander, J. J., et al., 1972; Englander & Von Hippel, 1972). Estimation of the number of slow exchangeable protons of nucleic acids provides a description of the tertiary structure of these molecules in solution (Englander & Englander, 1965). Furthermore, the exchange behavior reflects the time-dependent conformational fluctuations ("breathing") of the secondary and tertiary structure of nucleic acids (Printz & Von Hippel, 1965; Englander & Englander, 1965).

Pioneering work (Printz & Von Hippel, 1965; Englander & Englander, 1965) has been done by the gel filtration technique monitoring the tritium-hydrogen reexchange as a function of time (Englander & Englander, 1978). The corresponding deuterium-hydrogen reexchange has been monitored by several spectroscopic methods such as real-time nuclear magnetic resonance (Johnston & Redfield, 1979) and the stopped-flow technique in the ultraviolet region (Cross, 1975; Nakanishi et al., 1977; Nakanishi & Tsuboi, 1978). The time scale has been expanded by nuclear magnetic resonance techniques in the frequency domain (Marshall & Grunwald, 1969) and in the time domain (Johnston & Redfield, 1979), measuring the line width and the relaxation time, respectively.

Fundamental interpretation of the exchange processes of nucleic acid base protons in ordered structures is based upon the knowledge of the exchange kinetics of the mononucleotides or nucleosides. The NH_2 exchange of adenine, guanine, and cytosine nucleotides has been studied thoroughly (McConnell

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