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Biochemistry

lac repressor: 3-Fluorotyrosine substitution for nuclear magnetic resonance studies

(amino acid analogue/fluorine-19 nuclear magnetic resonance/nuclear spin label)

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ABSTRACT **This paper describes the isolation of 3-fluorotyrosine-substituted *lac* repressor, and its ^{19}F nuclear magnetic resonance spectrum. From the spectrum, one can conclude that for each of the four identical subunits of the repressor there are four or five surface tyrosines, two buried or internal tyrosines, and one tyrosine with an phenolic group ionized or involved in a hydrogen bond. Conditions are described that can be used for the 3-fluorotyrosine substitution of a variety of *Escherichia coli* proteins for ^{19}F nuclear magnetic resonance studies.**

Among the genetic control systems that are known to date, only the lactose operon system of *Escherichia coli* has been sufficiently investigated to the point where the chemical structures of all of the components are known (1–3). Of particular interest are the two macromolecular components of the system, the *lac* repressor protein and the *lac* operator DNA sequence. The task now is to correlate the chemical structures with their functions. We describe here a nuclear magnetic resonance (NMR) approach to this problem.

Recent advances in the development of high sensitivity and resolution Fourier transform (FT) NMR spectrometers have made this a powerful tool in the investigation of the conformation and dynamics of macromolecules in solution. The use of ^1H NMR to study proteins, particularly ones as large as the *lac* repressor, which has four identical subunits of 347 amino acids each (3), has been limited by the complexity of the spectra obtained and the difficulty of making specific correlations between the features in the spectrum that one obtains and specific nuclei in the macromolecule.

Sykes *et al.* (4), Browne and Otvos (5), and Anderson *et al.* (6) have shown that it is possible to isolate alkaline phosphatase and coliphage fd gene 5 protein that have been substituted with 3-fluorotyrosine. In both cases, the resulting ^{19}F NMR spectra show as many resolved peaks as tyrosines in the subunit polypeptide chain: eleven in the case of alkaline phosphatase and five in the case of gene 5 protein. As Sykes *et al.* point out, the use of fluorine in place of hydrogen as a spin probe yields both a simplification of the NMR spectrum and a greater chemical shift range. Unlike the other heavier nuclei used in NMR studies, ^{19}F is 100% abundant and there is only a 17% loss in sensitivity in comparison to ^1H NMR measurements. We show here that the incorporation of 3-fluorotyrosine as a method to circumvent the complexity of the ^1H NMR spectrum can be applied to the *lac* repressor.

This paper describes the efficient incorporation of 3-fluorotyrosine into *lac* repressor under conditions applicable to a number of interesting *E. coli* proteins, without the use of tyrosine-requiring strains (4–6). The addition of fluorine nuclei at the tyrosines does not drastically alter the properties of the *lac* repressor.

The *lac* repressor is particularly suited to this approach, since mutations that occur at each of the eight tyrosines (7, 8) have been isolated in the *lacI* gene. It will be possible to assign the features in the ^{19}F NMR spectrum to specific tyrosines in the *lac* repressor primary structure by isolating fluorotyrosine *lac* repressor from each of these mutants. This leads to a simple solution of the problem of NMR peak assignments; thus, a detailed NMR analysis of the solution structure and dynamics of the *lac* repressor molecule, including changes that occur upon interaction with other molecules, can be pursued with a precise knowledge of the amino acid residues involved.

MATERIALS AND METHODS

The *E. coli* strain used throughout these experiments is CSH46 (same as M96) (9). This strain requires proline and thiamine, is unable to grow on arabinose or lactose, and is lysogenic for a temperature-inducible prophage ($\lambda cI857$, St68, h80d*lacI,Z*) which in turn carries part of the *lac* operon. The *lac* region contains a mutation in the Z gene (U118) and has the *I* (SQ) overproducing promoter.

Media. Rich medium, when used, was LB (9). Minimal medium was M9 (9) supplemented with glucose (1%), thiamine (1 μg/ml) and the naturally occurring L amino acids (20 μg/ml, or 0.2 mM) except tyrosine, tryptophan, and phenylalanine. Tryptophan and phenylalanine were present at 1 mM.

DL-3-Fluorotyrosine was synthesized starting with 3-fluoro-*p*-anisaldehyde (Aldrich Chemical Co.) as described in ref. 10. The 3-fluorotyrosine obtained was characterized by its infrared and ultraviolet spectra, and by the ^{19}F NMR spectrum at neutral and alkaline pH. No tyrosine contamination could be found when the 3-fluorotyrosine was chromatographed on cellulose plates in isopropanol:NH_4OH (17%):H_2O, volume ratio 4:2:1, or electrophoresed at pH 9.2 in 0.05 M NH_4HCO_3 buffer on Whatman 3MM paper. Separation in the electrophoresis occurs because the pK of the 3-fluorotyrosine phenolic proton is 8 rather than 10.5 for tyrosine. 3-Fluoro[^3H]tyrosine and [^3H]tyrosine were made by exchanging the ^3H from ^3H_2O onto the α position (11).

Purification of the 3-fluorotyrosine *lac* repressor, isopropyl-β-D-thiogalactoside (IPTG) binding assays, and operator DNA binding assays follow published procedures for normal *lac* repressor (9, 12–14). The 3-fluorotyrosine *lac* repressor had about 35% activity for operator binding, comparable to published results with normal *lac* repressor (12, 13). The absorption coefficient was determined by comparing a biuret protein measurement (15) of the 3-fluorotyrosine *lac* repressor with a standard curve obtained for normal *lac* repressor. Both proteins were judged to be greater than 98% pure from gel electrophoresis (16). Overall yields of 3-fluorotyrosine *lac* repressor from the phosphocellulose step (12) have been about 40–50% of the

Abbreviations: NMR, nuclear magnetic resonance; FT, Fourier transform; IPTG, isopropyl-β-D-thiogalactoside.

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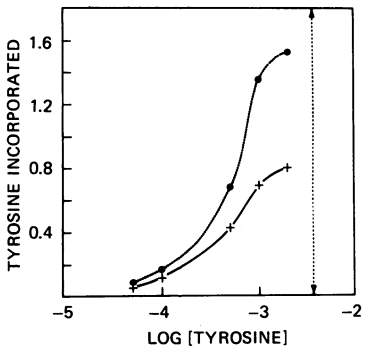


FIG. 1. Tyrosine and 3-fluorotyrosine incorporation as a function of concentration in the medium, under the conditions of prophage induction. The CSH 46 strain was grown to an optical density of 1.0 at 550 nm at 30° in 5 ml of M9 plus all of the amino acids except tyrosine as described in *Materials and Methods*. The culture was then shifted to a 42° water bath and DL-[α-^{3}H]tyrosine or DL-3-fluoro-[α-^{3}H]tyrosine was added to give the indicated concentration. After 25 min at 42° the temperature was lowered to 37° and the culture was incubated for a further 3 hr. The nanomoles of tyrosine and 3-fluorotyrosine per mg of protein were measured by scintillation counting and biuret determination (15). The dotted line with filled circles is for tyrosine incorporation and the solid line with crosses is the curve for fluorotyrosine incorporation. The arrow indicates the concentration of a saturated solution of tyrosine at 37° (27).

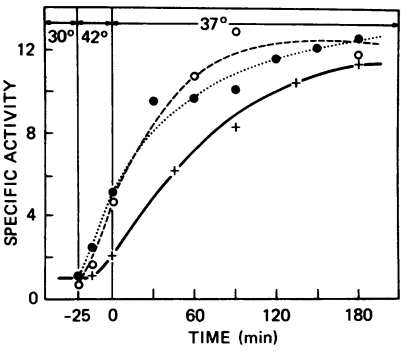


FIG. 2. Synthesis of *lac* repressor as a function of time in various media. This figure shows the basic protocol for the induction of the prophage. The cells containing the phage were cooled to 0° and harvested by centrifugation at 3 hr. 3-Fluorotyrosine, when required, was added at −25 min in the above figure. The cells were grown to an OD_{550} of 1.0 and the temperature was shifted as indicated at −25 min. The specific activity of *lac* repressor, 100 × ([IPTG-bound]/[IPTG-free]) ÷ (protein concentration in mg/ml), is measured by [^{14}C]IPTG binding with 10 ml aliquots of the culture as described in ref. 9. The dotted line with filled circles is for rich medium; the broken line with open circles, minimal medium with tyrosine added to 1 mM at −20 min; the solid line with crosses, minimal medium with 3-fluorotyrosine added to 1 mM at −20 min.

yield from equal amounts of bacteria grown in rich medium.

Percentage substitution of tyrosine by the 3-fluorotyrosine: *lac* repressor isolated from the bacteria induced in the presence of 3-fluorotyrosine was hydrolyzed in 6 M HCl at 120° for 18 hr. Tyrosine and 3-fluorotyrosine were separated from the rest of the amino acids by a 1 × 18 cm Bio-Rad AG 50W-X8 column eluted with 0.2 M pyridine acetate buffer at pH 3.1 (17). Tyrosine and 3-fluorotyrosine elute together, separated from the other amino acids. The fractions comprising the tyrosine peak from the column were then pooled, lyophilized, and redissolved in 0.01 M HCl and applied to Whatman 3MM paper for electrophoresis as described above. The tyrosine and fluorotyrosine regions were eluted from the paper with 0.01 M HCl and measured spectrophotometrically.

The ^{19}F FT-NMR spectra were obtained at 94 MHz with a JEOL model JNM-PS 100 FT-NMR spectrometer equipped with a 10 mm fluorine probe and a variable temperature attachment. An internal deuterium lock was used to assure field stability during data accumulation. The 3-fluorotyrosine *lac* repressor was dialyzed against the D_2O buffer at 4° in a dialysis block for 24 hr just prior to the NMR measurements. Other details are in the legend for Fig. 5.

RESULTS

Incorporation of the 3-Fluorotyrosine. In previous attempts to biosynthetically incorporate the fluorine nucleus into proteins for spectroscopic purposes, tyrosine (4–6) or tryptophan (5, 18) auxotrophs were used. Since Sykes *et al.* (4) have shown that the incorporation of 3-fluorotyrosine is a useful tool in studying protein structure and dynamics, it seemed worthwhile to develop a general method of getting this analogue into proteins without the necessity of constructing the tyrosine auxotroph in every instance.

Because 3-fluorotyrosine and 2-fluorotyrosine are growth inhibitors (19), it is not possible to simply grow the *E. coli* in a medium containing one or the other analogue. In the case of auxotrophs, since there is competition for the transfer RNA activating enzyme that very much favors the normal tyrosine (20), one must then arrange the growth conditions to have the bacterial culture exhaust the supply of tyrosine before the analogue is added. The alternative is to change the medium by filtration or centrifugation of the bacteria followed by resuspension. This is usually not practical when one considers the culture volumes necessary to obtain proteins in the amounts required by NMR measurements.

It should be possible to take advantage of the ability of tryptophan, phenylalanine, and 3-fluorotyrosine to feedback-inhibit and repress the first enzyme of the aromatic pathway, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase, which exists as three isoenzymes that are sensitive to each of the aromatic amino acids (20, 21). In *E. coli*, the form of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase sensitive to repression and feedback inhibition by tyrosine is undetectable in cells grown in the presence of the aromatic amino acids (22). This is reflected in an observation that 10 mM 3-fluorotyrosine in the medium reduces tyrosine incorporation by 96% in wild type *E. coli* (23). We chose 1 mM, since this allowed near maximal incorporation (see Fig. 1), and, at the same time, used 10-fold less 3-fluorotyrosine. The phenylalanine-sensitive form of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase is heat sensitive (24), so that it should be inactivated during the heat induction step we employ. Under these conditions, we can add the 3-fluorotyrosine in the middle of balanced exponential growth and take advantage of the bacterial protein synthesizing system while it is functioning optimally. This could be the reason that our yields of 3-fluorotyrosine *lac* repressor, 50%, are higher than the yields of 3-fluorotyrosine alkaline phosphatase, 10%, obtained by Sykes *et al.* (4).

Since the addition of the 3-fluorotyrosine is growth inhibitory, Sykes *et al.* (4) designed their growth medium to run out of phosphate at the same time the normal tyrosine in the medium was exhausted. Thus, the addition of the 3-fluorotyrosine coincided with the derepression or induction of alkaline phosphatase. This results in a higher differential rate of alkaline phosphatase synthesis. Alkaline phosphatase molecules without the analogue are only those made under repressed conditions. Here we take advantage of the fact that the *lac* repressor must be isolated from cells carrying a prophage, if one needs milligram amounts for biochemical experiments (25). The induction

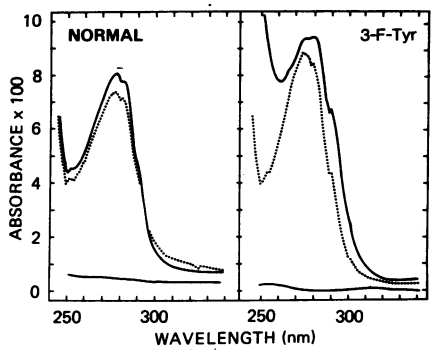


FIG. 3. The absorption spectra of *lac* repressor in 8 M guanidine·HCl. Normal and 3-fluorotyrosine (3-F-Tyr)-substituted *lac* repressor, both at about 100 μg/ml, were made up in solutions containing 8 M guanidine·HCl (Schwarz/Mann) and either 0.05 M citrate buffer or 0.1 M NH_4HCO_3 buffer. The dotted lines are for pH 6.9 and the solid lines are for pH 9.0, as measured by the pH meter in the 8 M guanidine·HCl solution. The base line is for solvent measured against solvent. These spectra were taken with a Cary 15 spectrophotometer.

and replication of the prophage increases the number of gene copies for the *lac* repressor for the purpose of 3-fluorotyrosine incorporation. Fig. 2 shows that not only is the rate of appearance of *lac* repressor about the same as in rich medium or minimal medium without 3-fluorotyrosine, but also the same specific activities for IPTG binding are reached in the presence of the analogue.

Properties of the 3-Fluorotyrosine-Substituted Repressor. Table 1 summarizes and compares some of the physical properties of the *lac* repressor isolated from the cells grown in minimal medium and induced in the presence of 3-fluorotyrosine with the properties of normal *lac* repressor. We note here a somewhat higher extinction coefficient as was seen in the case of 3-fluorotyrosine alkaline phosphatase (4). Fig. 3 shows the spectroscopic changes at two different pH values under denaturing conditions. The 3-fluorotyrosine-substituted *lac* repressor undergoes large changes in the absorption spectrum when shifted from pH 7 to pH 9, due to the fluorine substitution. Using the assumption that extinction coefficients (27, 28) are additive, the hypochromicity in Fig. 3 shows that the 3-fluorotyrosine *lac* repressor has about 90% of its tyrosines replaced by the fluorine analogue. This agrees with the data in Table 1 obtained by amino acid analysis.

The ability of the 3-fluorotyrosine *lac* repressor to bind inducer, IPTG, and operator-containing DNA is implied by the measurements of the binding constant for IPTG and the dissociation constant for the *lac* repressor-operator complex in Table 1. The interaction with operator DNA is sensitive to IPTG. These results suggest that the incorporation of 3-fluorotyrosine has not significantly altered the structure and function of the *lac* repressor. The difference between our measurements of the dissociation rate constant of *lac* repressor from operator DNA is not very significant, since we see this type of variation in comparing different preparations of normal *lac* repressor.

NMR Spectrum. Fig. 4 shows the ^{19}F NMR spectrum of the native 3-fluorotyrosine *lac* repressor in Tris–KCl buffer. Fig. 5 shows the ^{19}F NMR spectrum of the *lac* repressor in the same buffer with 8 M guanidine·HCl. One notes that the six resonances in Fig. 4 are reduced to a single resonance in Fig. 5. This result is similar to that observed by Sykes *et al.* for alkaline phosphatase (4), and indicates that the chemical shifts and different linewidths of the six resonances seen in Fig. 4 arise from fluorines on tyrosines in different environments with different relative freedoms of motion. The spectrum shown in Fig. 4 was taken in the presence of 1 mM IPTG, and represents *lac* repressor complexed with one IPTG per subunit. The positions indicated by *D* and *F* in Fig. 4 show the peak position for the single peak in the denatured *lac* repressor spectrum and the position of the center of the resonance peaks for fluorotyrosine at pH 10, respectively. The center of the resonance peaks for 3-fluorotyrosine at pH 7.5 is identical to the position of the resonance seen in Fig. 5. Although the spectrum of free fluorotyrosine is more complex due to coupling with the ^1H nuclei in the phenol ring, the width of the spectrum of free fluorotyrosine (0.25 ppm) is less than the width of a single resonance peak in Fig. 4. [There is a negative nuclear Overhauser enhancement in alkaline phosphatase (4), so that proton decoupling was not attempted.] The decreased freedom of motion of fluorotyrosine when it is incorporated in a large polypeptide chain results in a broadened peak for the fluorine resonance. A theoretical treatment by Hull and Sykes (29, 30) shows that one may make a rough estimate of the number of fluorines and thus the number of tyrosines from an integration of the NMR spectrum made under the conditions described in the legend to Fig. 4. The areas of the peaks normalized to ½ of the area of peaks 1 plus 2 is shown in Table 2. Not all of the resonances need have their full intensities represented here, since it is possible that the delay between transients in the accumulated spectrum was not long enough. From the sum seen in the table we can account for 7.5 of the eight tyrosines in the *lac* repressor subunit.

Table 1. Properties of normal and 3-fluorotyrosine-substituted *lac* repressor

	Normal	Substituted
Percentage tyrosine*	100	9.8
Percentage·fluorotyrosine*	—	90
$A_{280}^{0.1\%}$	0.60	0.67
K_d for IPTG (4°), μM	1 (± 20%)	1 (± 20%)
k_d† for operator, sec^{-1}	$2–3 \times 10^{-4}$	$3–4 \times 10^{-4}$

* This is percentage tyrosine or 3-fluorotyrosine in the tyrosine plus 3-fluorotyrosine fraction of the total acid hydrolysis.

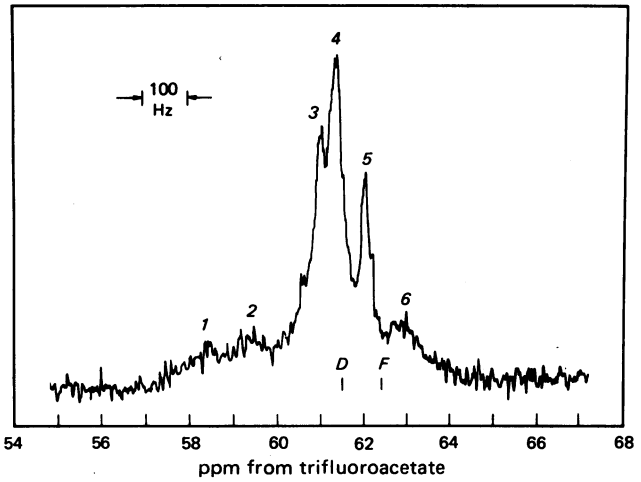
† k_d is the dissociation rate constant of the *lac* repressor·*plac* 5 DNA (operator DNA) complex at 25°. The wild-type value measured by Riggs *et al.* (26) is 6.2×10^{-4} sec^{-1}.

DISCUSSION

The data in Table 1 indicate that the properties of the *lac* repressor with tyrosines substituted with 3-fluorotyrosine are not significantly altered from the properties of the normal form. From the work done with suppressed nonsense mutants in the *lacI* gene (7), substitutions of tyrosine by other amino acids at positions 7, 12, 126, and 194 yield functional repressor. The presence of tyrosines, at positions 17, 47, 260, and 269 in the

Table 2. Relative areas of the resonance in Fig. 4 ± 10% (estimated error)

Peak	Relative area
1 + 2	2
3 + 4	3.5
5	1
6	1
Sum	7.5
No. of tyrosines in *lac* repressor subunit	8



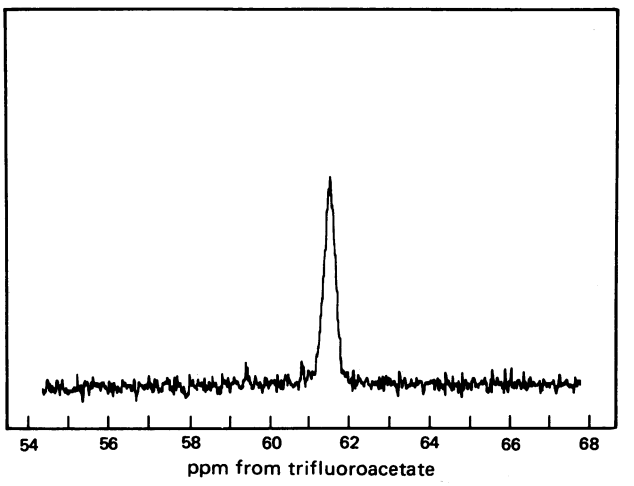


FIG. 4. (*left*) ^{19}F-FT-NMR spectrum of a solution of 3-fluorotyrosine *lac* repressor (30 mg/ml) at 37° in 0.2 M Tris·HCl, 0.2 M KCl, 0.3 mM dithiothreitol, and 0.1 mM EDTA in D_2O. The pH meter reading was 7.5 at 37°. The solution was 0.2 mM in tetramers (normal quaternary structure) or 0.8 mM in subunits. IPTG concentration was 1 mM. The spectrum is the result of averaging 90 blocks of 500 transients each. An 80° pulse was used with a 1.2 sec pulse repetition time. The chemical shifts are measured from the resonance of trifluoroacetate added as an internal chemical shift standard. The *D* on the scale indicates the position of the single peak from denatured *lac* repressor; the *F* denotes the center of resonance peaks of 3-fluorotyrosine at pH 10.

FIG. 5. (*right*) ^{19}F-FT-NMR spectrum of the 3-fluorotyrosine *lac* repressor from Fig. 4 in 8 M guanidine·HCl in D_2O at a pH meter reading of 8. Trifluoroacetate was added as an internal standard. The spectrum required 28 blocks of 500 transients each. An 80° pulse and 2.6 sec repetition rate were used. The sample temperature was 37°.

amino acid sequence, is essential for functional repressor. The results here show that fluorine insertion at these residues does not significantly alter the properties of the repressor.

Our observation here is consistent with the observations that β-galactosidase can tolerate the incorporation of *p*-fluorophenylalanine (31), *o*-fluorophenylalanine (31), as well as 3-fluorotyrosine (23), and fluorotryptophan (17); and that active alkaline phosphatase can be formed with *p*, *o*, or *m*-fluorophenylalanines (31), as well as 3-fluorotyrosine. The van der Waals radius of the fluorine atom is 1.35 Å compared with 1.2 Å for hydrogen (32). Thus, 3-fluorotyrosine should not have a large effect on the structure of a protein, although it may have a large effect on catalysis if it were in the active site, since its pK is altered.

It is possible to make several observations about the *lac* repressor tyrosines from the NMR spectrum shown in Fig. 4, even without the assignment of the peaks. As Sykes *et al.* observed in alkaline phosphatase, the peaks shifted the most downfield have the greatest line widths (4). The difference in chemical shift reported by Sykes *et al.* for the denatured alkaline phosphatase resonance, about 58 ppm (4), and that for the *lac* repressor shown in Fig. 5, about 61.5 ppm, is probably due to their use of an external standard and our use of an internal standard. From their extensive analysis of the correlation of chemical shift with both the relaxation times (33) and the solvent isotope shift (34), one can say that the tyrosines represented by peaks 1 and 2 are buried in the protein with restricted motion. Peak 6 is a broad peak and is an exception in that it occurs on the highfield side of the spectrum. Its position nearly corresponds to the position of a tyrosine where the phenolic proton has dissociated. A possible explanation is that this fluorotyrosine side chain is hindered in its motion with respect to the *lac* repressor molecule and at the same time is ionized or involved in a hydrogen bond. The remaining 4.5 to five tyrosine resonances are near the single resonance peak position for the denatured protein. Thus, these tyrosines are likely to be on the surface of the protein in contact with the solvent where there are fewer constraints on their motion. This conclusion is in agreement with experiments that result in iodination (35) or acetylation and nitration of native *lac* repressor (K. Matthews, personal communication). There are some small differences in the NMR spectra taken with *o*-nitrophenyl fucoside, an anti-inducer, and in the absence of IPTG, suggesting changes in the environments at the tyrosine side chains. More extensive measurements are required to determine their significance.

As we mentioned in the introduction, one reason for picking the *lac* repressor for this study is the existence of nonsense mutations that have been shown to be at the eight tyrosine residues in the amino acid sequence (7, 8). The isolation and purification of *lac* repressors from these mutant *lacI* genes in a nonsense suppressor strain which inserts a substitute for tyrosine, one at a time, would allow the positive correlation of the peaks that we see in Fig. 4 to specific tyrosines in the amino acid sequence. One would expect single peaks to be missing from the spectra of the 3-fluorotyrosine *lac* repressors isolated from the mutants. Such positive assignments will allow one to use the analysis that Hull and Sykes (29, 30, 33, 34) have developed for ^{19}F NMR spectroscopy at specific points in the *lac* repressor primary structure. This application of suppressed nonsense mutations for identification and assignment of spectral changes to specific amino acids residues has already been exploited in the fluorescence emission spectrum of the *lac* repressor (13). An additional reason for picking this system is the fact that Miller and coworkers have isolated and characterized 79 nonsense mutations in the *lac* repressor subunit sequence of 347 amino acids (7). With the use of tyrosine-inserting nonsense suppressors it will be feasible to insert a 3-fluorotyrosine in at least 71 additional sites in the protein, one at a time, if any other parts of the *lac* repressor sequence merit closer analysis. (Eight of the 79 sites are already represented by the spectrum shown in Fig. 4.)

Sykes *et al.* have shown that ^{19}F NMR analysis of alkaline phosphatase is a valuable and informative probe of the conformation and dynamics of that enzyme. The results we present here show that ^{19}F NMR can yield meaningful information about the *lac* repressor as well. How general will this approach be? The conditions we have used to incorporate 3-fluorotyrosine should be generally applicable. The *lac* repressor was isolated

from a strain that does not require tyrosine for growth—an important technical point since the use of tyrosine auxotrophs forces one to employ either tyrosine starvation conditions (4) or centrifugation with resuspension (5, 18). The conditions that we present give both high yields of the desired protein and higher levels of analogue substitution. Sykes *et al.* (4) obtained a 10% yield of substituted alkaline phosphatase and 73% substitution of the tyrosines. Browne and Otvos (5) achieved normal yields with 75% substitution with this protein. Anderson *et al.* (6) reported a 15% yield of gene 5 protein from *E. coli* infected with coliphage fd when compared with normal procedures. Pratt and Ho (18), in using a variety of fluorotryptophan analogues for the introduction of the ^{19}F nucleus, found 51–73% substitution with a tryptophan auxotropy. Our results with the *lac* repressor indicate that the yields can be 40–50% when compared to the normal protein and we can achieve substitution rate of 90% for tyrosine. Initial experiments with tryptophan analogues indicate the same procedures can also be applied for that amino acid.

One requirement for the success of any of these procedures is the induction of the protein of interest so that a higher differential rate of synthesis occurs during exposure to the fluoro analogue. In the case of the *lac* repressor, this was accomplished through the use of an inducible prophage. This implies that a number of other interesting, normally uninducible systems are also amenable to the use of ^{19}F NMR for structural studies. Those proteins whose structural genes are already on similar prophages include the regulatory gene products of the galactose operon (36), the arabinose operon (37), the tryptophan operon (38), and the histidine utilization operon (39); the α, β, and β' subunits of RNA polymerase (40, 41); elongation factors T_u and G (41); a number of ribosomal proteins (41); and, of course, the repressor of λ phage along with its early control proteins.

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