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Inducer and Anti-inducer Interactions with the *lac* Repressor Seen by Nuclear Magnetic Resonance Changes at Tyrosines and Tryptophans*

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Frank Boschelli, Mary Ann C. Jarema‡, and Ponzy Lu§

From the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The effects of binding inducer and anti-inducer of the *Escherichia coli lac* operon to the *lac* repressor were examined by taking advantage of fluorine-19 NMR. The fluorine nucleus was biosynthetically incorporated into the *lac* repressor with either 5-fluorotryptophan or 3-fluorotyrosine. It is suggested that these small effector molecules influence the operator-binding properties of the tetrameric *lac* repressor by altering the intersubunit relationships in the protein.

Even before being confirmed as a protein (Gilbert and Müller-Hill, 1966, 1967), the *lac* operon repressor of *Escherichia coli* was counted among "allosteric proteins, those most elaborate products of molecular evolution" (Monod *et al.*, 1963). This protein, with four identical subunits of 360 amino acid residues each, binds the *lac* operator DNA sequence preventing the expression of the *lac* operon (Bourgeois and Pfahl, 1976). Allosteric inducers, allolactose in nature (Jobe *et al.*, 1972), isopropyl β-D-thiogalactoside, in the laboratory, accelerate the rate of dissociation of the repressor from the operator DNA (Riggs *et al.*, 1970). Anti-inducers, analogues of inducers, increase the lifetime of the *lac* repressor-*lac* operator-DNA complex (Riggs *et al.*, 1970). Among the latter group are phenyl or nitrophenyl galactosides and fucosides as well as glucose and lactose (Barkley *et al.*, 1975).

Using spectroscopic methods, considerable attention has been focused on the interaction of inducer molecules, especially IPTG,[1] with the *lac* repressor (Laiken *et al.*, 1972; Matthews *et al.*, 1973; Wu *et al.*, 1976). As might be expected, because spectroscopic parameters undergo changes when IPTG is bound, most of the above authors postulate allosteric or conformational changes in *lac* repressor structure. The anti-inducer binding site overlaps, at least partially, the inducer binding site on the protein as shown by competition studies with IPTG (Barkley *et al.*, 1975). It has been difficult to study the binding of anti-inducers containing highly UV absorbing substituted phenyl groups along with much weaker binding constants (greater than 10^{-4} M measured by competition with IPTG) by optical spectroscopic methods due to interference with the intrinsic chromophores such as tyrosines or tryptophans. The binding constants of the nonabsorbing anti-inducers, glucose and lactose, are so low (14 M^{-1} and 3 M^{-1}, respectively) (Barkley *et al.*, 1975) that solution viscosity problems arise if the effector concentration is high enough to saturate the binding sites. ^{1}H-NMR observations on the protein have been severely hampered by the large size of the tetrameric protein (154,520 daltons) which leads to broad, unresolved resonances, even in the aromatic region (Buck *et al.*, 1978; Wade-Jardetzky *et al.*, 1979).

We have taken a combined genetic (Miller *et al.*, 1979) and NMR approach to the examination (Jarema *et al.*, 1981b; Arndt *et al.*, 1981) of the structure-function relationships in the *lac* repressor, and have been able to overcome some of the limitations of the earlier work in the study of the interaction of anti-inducer with the protein. We describe here the ^{19}F-NMR spectrum of *lac* repressor where the 2 tryptophan residues have been replaced by 5-fluorotryptophan. We have assigned the two resulting fluorine resonances with the suppressed nonsense codon at position 201 in the *lacI* gene in a manner similar to what we have done in the case of 3-fluorotyrosine-substituted repressor (Jarema *et al.*, 1981b). Having assigned the resonances, we can monitor the tryptophan residue that undergoes environmental changes upon binding an anti-inducer molecule.

We show here that significant environmental changes occur only at tryptophan 220 and not at 201 when the inducer, IPTG, or the anti-inducer, ONPF, are bound. The same tryptophan shows changes in both NMR and fluorescence properties (Sommer *et al.*, 1976) upon IPTG binding. The precise environment change at tryptophan 220 cannot be identical for both ligands. Examination and comparison of the ^{19}F-NMR spectrum of 3-fluorotyrosine-substituted *lac* repressor shows that IPTG and ONPF have very different effects when monitored at the tyrosines.

MATERIALS AND METHODS

3-Fluorotyrosine was synthesized from *o*-anisidine (English *et al.*, 1940; Kraft, 1951; Dibbo *et al.*, 1961). Incorporation of the 5-fluorotryptophan analogue in the *lac* repressors followed exactly the procedures we have used for 3-fluorotyrosine, except that the 5-fluorotryptophan was substituted for 3-fluorotyrosine and L-tyrosine and L-phenylalanine were used for feedback inhibition of the aromatic pathway (Lu *et al.*, 1976).

Isolation and purification of the *lac* repressors from both wild type and the mutant strains followed procedures we have published (Sommer *et al.*, 1976; Lu *et al.*, 1976; Jarema *et al.*, 1981b).

Isolation of *lac* repressor tetrameric cores followed published procedures (Platt *et al.*, 1978). Protein concentrations were determined using the following A_{280} for 1 mg/ml: *lac* repressor and 5-fluorotryptophan repressor, 0.60; 3-fluorotyrosine *lac* repressor, 0.53; 3-fluorotyrosine tetrameric core, 0.46. ONPF was purchased from Cyclo; IPTG and 5-fluorotryptophan were purchased from Sigma. Operator DNA binding and dissociation rates of 5-fluorotryptophan-substituted *lac* repressor were done with pOP 203-1 plasmid DNA containing the operator, end-labeled with ^{32}P via polynucleotide kinase (Lillis, 1981). NMR spectra were obtained on a Nicolet NT 150 spectrometer under conditions described in the figure legends. A 20 mm sample tube was used and chemical shifts are referenced to an external sample of 80

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‡ Present address, Department of Biochemistry, Brandeis University, Waltham, MA 02254.

§ To whom correspondence should be addressed.

[1] The abbreviations used are: IPTG, isopropyl β-D-thiogalactoside; ONPF, *o*-nitrophenylfucoside.

mM trifluoroacetic acid in 25% D_2O. The sweep widths reported are those on either side of the quadrature phase detector. Fluorescence measurements were obtained from a Perkin Elmer MPF4 spectrofluorometer.

RESULTS

Before considering the NMR spectra and the changes upon binding ONPF, we wished to be certain that the substitution with 5-fluorotryptophan in the wild type *lac* repressor does not significantly affect its biochemical properties. We have described the properties of tyrosine-substituted *lac* repressor and noted that it binds *lac* operator DNA and releases that DNA upon binding IPTG in a manner not very different from the native repressor (Lu *et al.*, 1976). In the course of this work we found that the same applies for the 5-fluorotryptophan-substituted *lac* repressor. In particular, ONPF increases the lifetime of the *lac*-repressor, *lac*-operator complex, with either 3-fluorotyrosine or 5-fluorotryptophan substituted in the protein. These data are shown in Table I. We have also looked at the effect of IPTG on the emission spectrum of fluorotryptophan repressor. The emission maxima of the 5-fluorotryptophan repressor is red-shifted about 10 nm from unsubstituted repressor to 351 nm. The binding of IPTG to this repressor causes a blue shift of 9 nm, comparable to that seen for normal repressor (Laiken *et al.*, 1972; Sommer *et al.*, 1976).

Fig. 1*A* shows the ^{19}F-NMR spectrum of the *lac* repressor containing 5-fluorotryptophan. There are two resonances representing the two tryptophans per subunit in the *lac* repressor (Farabaugh 1978; Beyreuther, 1978). The spectrum shows that there is probably no major asymmetry in the environment of each of the tryptophans in the four subunits.

The assignment of the resonances, due to the 2 tryptophans of repressor, was based on comparison of the spectrum of an altered repressor from a strain with a tyrosine substitution at tryptophan position 201 (the substitution was by suppressing a nonsense mutation, Sommer *et al.*, 1976) and on the effect of the binding of the inducer IPTG on the spectrum of wild type repressor with 5-fluorotryptophan. It should be noted that the amino acid sequence of the *lac* repressor was corrected in 1978 (Farabaugh, 1978; Beyreuther, 1978). With these corrections, what was formerly labeled tryptophan 190 becomes tryptophan 201 and tryptophan 209 becomes tryptophan 220. We are using the corrected numbering when referring to all previously published work.

Fig. 1*B* shows the ^{19}F NMR spectrum of the altered repressor which has a tyrosine for tryptophan substitution at residue 201. One anticipates that one of the two resonances would be removed and that the other resonance would be unchanged if the structure of the altered repressor were similar to native repressor. Fig. 1*B* shows that there may be structural alterations in the vicinity of the remaining tryptophan as shown by the three resonances in the spectrum of this altered repressor. One clearly corresponds to the resonance seen in the downfield region of the spectrum of wild type repressor. The middle resonance has a chemical shift similar to that seen for urea-denatured repressor and also corresponds to the chemical shift of the minor resonance seen in the spectrum of wild type repressor slightly upfield of the most downfield resonance. There is a possibility that both this resonance and the smaller resonance upfield are due to some conformational heterogeneity in the environment of this tryptophan in the altered repressor. We assign the upfield resonance in the wild type repressor spectrum to tryptophan 201 which is missing in the altered repressor spectrum and the downfield resonance to tryptophan 220.

TABLE I

Dissociation rate constants of fluoro-substituted lac repressor-lac operator complexes

	k_D −ONPF	k'_D +ONPF	k_D/k'_d
5-Fluorotryptophan repressor	8×10^{-4} s^{-1} $t_{1/2}$ = 14 min	3.5×10^{-4} s^{-1} $t_{1/2}$ = 33 min	2.3
3-Fluorotyrosine repressor	10×10^{-4} s^{-1} $t_{1/2}$ = 12 min	4×10^{-4} s^{-1} $t_{1/2}$ = 26 min	2.5
Wild type repressor (Riggs *et al.*, 1970)	6×10^{-4} s^{-1} $t_{1/2}$ = 19 min	2.3×10^{-4} s^{-1} $t_{1/2}$ = 50 min	2.6

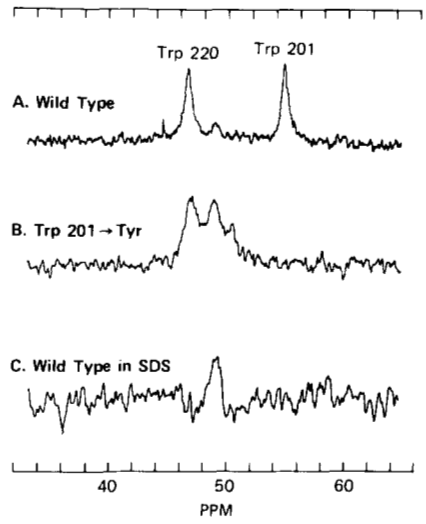

FIG. 1. **^{19}F-NMR spectra of 5-fluorotryptophan-labeled repressor.** The sweep width was 10,000 Hz and 8192 data points were collected with an acquisition time of 0.2 s. A line-broadening of 15 Hz was applied. The buffer was 0.1 M triethanolamine-bicarbonate, 0.1 M KCl, 3×10^{-3} M dithiothreitol, 10^{-4} M EDTA, 25% D_2O, pH meter reading 7.5 at room temperature. *A*, wild type repressor at 1.6×10^{-5} M. Recycle delay = 1.2 s. 53,200 scans were transformed. *B*, repressor with tyrosine for tryptophan substitution at position 220. The protein concentration was 9.7×10^{-6} M. Recycle delay = 0.7 s: 112,000 scans were collected. *C*, same as *A*, but denatured in sodium dodecyl sulfate, 7 M urea.

To confirm this assignment, we recall that binding IPTG to the wild type *lac* repressor causes a change in the environment of tryptophan 220 (Sommer *et al.*, 1976). This observation was derived by looking at tryptophan fluorescence spectra in the wild type and the two altered repressors where tryptophan was replaced by tyrosine via suppression of individual nonsense mutations (Sommer *et al.*, 1976). We noted above that the same change in the fluorescence spectrum occurs in the fluorotryptophan repressor. In addition, Bandyopadhyay and Wu (1979) and Brochon *et al.* (1977) showed that IPTG mainly affects the excited state lifetime of tryptophan 220. Fig. 2 shows the effect of inducer binding on the spectrum of 5-fluorotryptophan-substituted wild type repressor. A very large shift (3 ppm) in the downfield resonance is seen whereas only a relatively small change is seen in the upfield resonance. These results confirm the assignment of the downfield resonance to tryptophan 220.

Upon addition of ONPF to a concentration of 10^{-3} M, the resonance that we have assigned to tryptophan 220 is shifted downfield almost 2 ppm whereas only a small change is seen in the resonance resulting from tryptophan 201. Thus, it appears that ONPF, like IPTG, affects mainly the environment of tryptophan 220. Titration of 5-fluorotryptophan-substituted repressor with ONPF, at concentrations where both bound and unbound repressor coexist, shows two downfield peaks corresponding to repressor with and without ONPF. Thus, bound ONPF, like IPTG (Jarema, 1981), is in slow exchange with free ligand.

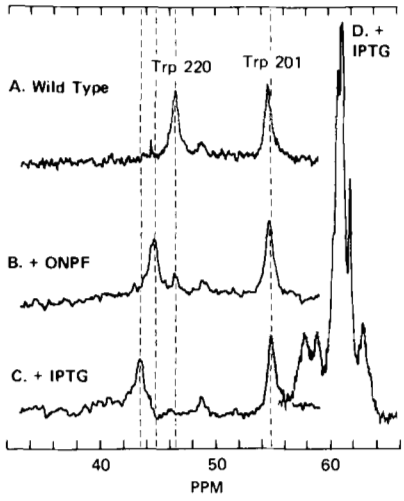

FIG. 2. **^{19}F-NMR spectra of fluorine-labeled repressor.** *A*, the spectrum of fluorotryptophan-labeled repressor in Fig. 1*A*; *B*, same as *A*, but in the presence of 10^{-3} M ONPF; 34,000 scans were collected; *C*, same as *A*, but with 10^{-4} M IPTG; 38,000 scans. *D*, 3-fluorotyrosine-labeled repressor at 2.6×10^{-5} M with 10^{-4} M IPTG in 0.25 M Tris, 0.20 M KCl, 3×10^{-3} M dithiothreitol, 10^{-4} M EDTA, 25% D_2O, pH meter reading 7.4. A 5,000 Hz sweep width was used. The recycle delay was 0.509 s and 20,000 scans were collected. The line-broadening was 10 Hz. This spectrum is scaled so that the relative intensities of the core tyrosine resonances are comparable to the tryptophan resonance intensities.

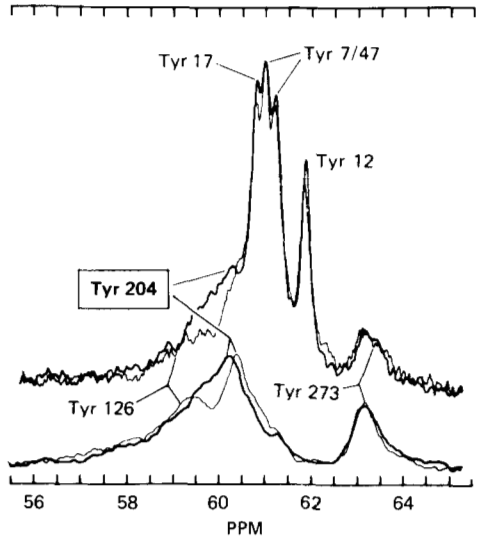

FIG. 3. **^{19}F-NMR spectra of 3-fluorotyrosine-substituted intact repressor and repressor core in the presence and absence of ONPF.** The assignments have been described previously (Jarema *et al.*, 1981). In each set of spectra the thin line is in the absence of ligand and the bold is in the presence of 10^{-3} M ONPF. The upper set of spectra are of intact *lac* repressor. The sweep width was 10,000 Hz and the acquisition time was 0.409 s. 16,000 data points were used. recycle delay = 1,409 s. A line broadening of 5 Hz was used. *Light spectrum*: 2.6×10^{-5} M repressor, 13,000 scans. *Bold spectrum*: 1.2×10^{-5} M repressor, 35,000 scans. The lower set of spectra are from repressor core. A 5,000-Hz sweep width was used. Acquisition time = 0.409 s, recycle delay = 0.509 s. The repressor concentration was 4.5×10^{-5} M. The buffer in each case was that described in Fig. 2*D* except the pH meter reading was 8.5.

The addition of ONPF at the same concentration to fluorotyrosine-substituted repressor results in the changes in the ^{19}F-NMR spectrum shown in Fig. 3. The upper pair of spectra are from the intact *lac* repressor, with the thick line the spectrum taken in the presence of ONPF and the thin line the spectrum of repressor without ONPF. The lower pair of spectra show the effect of ONPF on the spectrum of the tetrameric tryptic core. Platt *et al.* (1973) showed that it is possible to obtain four NH_2-terminal fragments consisting of the first 59 amino acids and a tetrameric core by limited tryptic digestion. The core contains the small sugar binding sites whereas the NH_2-terminal fragments show both specific and nonspecific DNA binding. We have previously shown that the NH_2-terminal headpiece and the tetrameric core yield ^{19}F-NMR spectra that add together to yield the spectrum of whole repressor (Jarema *et al.*, 1981). Only tyrosine 204 is affected by ONPF interaction to a significant degree. The assignment of the shoulder at 60 ppm in the presence of ONPF is from Jarema *et al.* (1981) along with the other resonance assignments.

Fig. 4 shows similar data (from Jarema *et al.*, 1981) for the interaction with IPTG for comparison. A large downfield shift (about 3 ppm) is seen for the resonance assigned to tyrosine 282. Tyrosine 204 is shifted upfield slightly while the other tyrosines are relatively unaffected by the binding of IPTG.

DISCUSSION

Examination of the static fluorescence emission spectra of both wild type and altered *lac* repressor with single tyrosine substitutions at each of the tryptophans respectively lead to the conclusion that the only change in the tryptophan environment is at residue 220 (Sommer *et al.*, 1976). This is consistent with the data shown here. Excited state lifetime measurements of the tryptophans of *lac* repressor (Brochon *et al.*, 1977, Bandyopadhyay and Wu, 1979) showed that the emission of tryptophan 220 is quenched by IPTG, while Brochon *et al.* (1977) saw an additional small change in the lifetime of tryptophan 201. This is consistent with our results, since we see a large downfield chemical shift of the resonance from tryptophan 220 and a slight upfield shift in the resonance assigned to tryptophan 201.

Bandyopadhyay and Wu (1979) also suggested that tryptophan 220 exists in two conformational states, because of

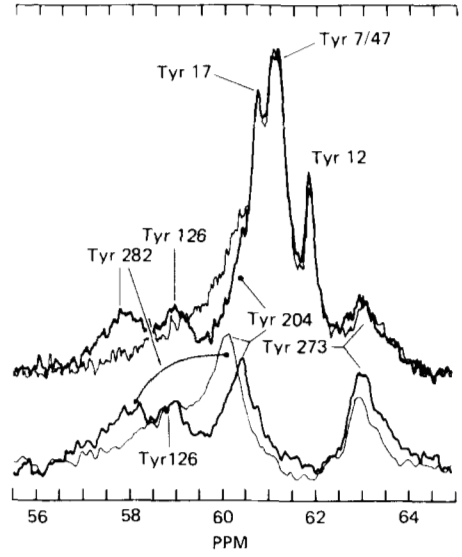

FIG. 4. **^{19}F-NMR spectra of 3-fluorotyrosine intact *lac* repressor (*upper set*) and tetrameric repressor core (*lower set*) in the presence and absence of IPTG.** In each set of spectra, the thin line is in the absence of ligand and the bold is in the presence of IPTG at 2×10^{-4} M. The buffer and NMR parameters are described in the legend to Fig. 2*D*. 20,000 scans were collected for the intact repressor spectra and 100,000 for the core spectra. The *wavy line* in the core spectra indicates the shift of the tyrosine 282 resonance that occurs upon IPTG binding.

either subunit heterogeneity or fluctuation of states, based on studies of the fluorescence emission from the repressor with a tryptophan at 220 only. The transient emission from this altered repressor which contains only one tryptophan showed two emission lifetimes with relative amplitudes of 60% and 40%, where the lesser component corresponded to the emission lifetime of tryptophan 220 native repressor. Also, a blue shift in the static emission spectrum upon quenching with acrylamide occurred which indicated that heterogeneous emitters exist in the repressor with tryptophan 220 only. The results presented here show that the tryptophans of native repressor do not fluctuate between two very different conformations on the NMR time scale since only one peak is seen for each tryptophan. However small fluctuations in the local environment of the tryptophans cannot be ruled out since the resonances are quite broad. The ^{19}F-NMR spectrum of the repressor with tryptophan 220 only (Fig. 1*B*) indicates, however, that the tryptophan in this altered protein may exist in several states. The possibility that one of these states is due to denatured or aggregated repressor is suggested by the similar chemical shifts of the middle resonance and the resonance of repressor denatured in 7 M urea. Bandyopadhyay and Wu showed that transient fluorescence of this altered repressor when IPTG is bound had, on the other hand, only one lifetime. This might be due to ligand stabilization of the repressor. The altered repressor with tryptophan at 220 only is temperature-sensitive *in vivo* suggesting that it is less stable than wild type repressor and may be why its NMR spectrum has several components (Miller *et al.*, 1979).

From Fig. 2, *C* and *D*, we note that the line widths for both the tryptophans and core tyrosines (see also Fig. 4) are quite comparable, about 1 ppm. For 3-fluorotyrosines having this line width, Hull and Sykes (1975) have estimated rotational correlation times in the range of 100 to 1000 ns for several resonances. Bandyopadhyay *et al.* (1981) have measured the rotational correlation time for the *lac* repressor tryptophans with and without inducer (IPTG) or anti-inducer (glucose). Superimposed on the 70-ns correlation time for the motion of the entire protein they see a fast component of 7 ns with IPTG and 10 ns without. Considering the fact that the 5-fluorotryptophan line widths are as broad as the broadest tyrosine line width, the rotational correlation times from these NMR data can be as much as 2 orders of magnitude larger than those measured by fluorescence experiments. The ^{19}F[^{1}H]nuclear Overhauser enhancements for both tryptophans are also essentially −1 as is the case for the core tyrosines, reflecting the immobilization of the residues. It will be possible to make more precise estimates of the rotational correlation time with the determination of line widths and longitudinal relaxation times at several magnetic fields, strengths as well as line width measurements at various temperatures to determine whether exchange broadening is a factor. Although fluctuation between conformations cannot be completely ruled out at this stage, experiments are in progress to see if this discrepancy between NMR and fluorescence experiments really exists. It is possible that the fluorescence depolarization measurements of the mobility of a fluorophore (Munro *et al.*, 1979; Bandyopadhyay *et al.*, 1981) may be observing motion induced by the excitation and emission processes.

The summary in Table II shows that: 1) it is not possible to state that inducer and anti-inducer interact at identical sites on the *lac* repressor; 2) both effectors cause changes in the physical status of tryptophan 220. A much smaller but similar change in the properties of tryptophan 201 occurs upon repressor binding to both ligands. IPTG and ONPF affect the chemical shift of tryptophan 220 differently. This is in agreement with Alexander *et al.* (1977) who showed that the IPTG molecule protected tyrosine 204, which may be near tryptophan 201, from nitration but ONPF did not and O'Gorman and Matthews (1977) showed that the inducer protected tryptophan 220 from oxidation by *N*-bromosuccinimide while ONPF did not.

TABLE II

Summary of chemical shift changes at the tryptophans and tyrosines (Δppm)

	+IPTG	+ONPF	Remarks
Tyr 7, 12, 17, 47	0	0	No effect on NH_2-terminal ⅓ of sequence by either ligand.
Tyr 126	0	0?[a]	Small negative shift possible with ONPF.
Tyr 204	+0.5	−1.5	Change of residue here influences Tyr 273 chemical shift (1)[b]; protected from nitration by IPTG (5); shift with IPTG measured using core.
Trp 201	+0.3	+0.3	
Trp 220	−3.0	−2.0	Near quaternary structure determinant at about residue 224 (3, 4).
Tyr 273	0	0	Change of residue here yields i^n, i^r (2, 5); possibly in hydrogen bond (1); nitrated (6)
Tyr 282	−3.0	0?	Tyr needed for quaternary structure (3); not nitrated (6).

[a] The 0? means a small, less than 0.3 ppm, shift may occur.

[b] Numbers in parentheses are references. (1) Jarema *et al.* (1981); (2) Miller *et al.* (1979); (3) Schmitz *et al.* (1976); (4) Miller (1980); (5) Myers and Sadler (1971); (6) Alexander *et al.* (1977).

A comparison of the spectral changes at tryptophan 220 and tyrosine 282 (Fig. 4) provides an interesting suggestion about the nature of the effect of inducer (IPTG). Both residues show a dramatic downfield shift of about 3 ppm upon binding of inducer to repressor. This is significant since both tyrosine 282 and the vicinity around tryptophan 220 are quaternary structure determinants of repressor. Schmitz *et al.* (1976) showed that a repressor variant with a serine substitution at tyrosine 282 results in a repressor which loses the ability to form tetramers. There is a missense mutation at a position just past tryptophan 220 affecting the quaternary structure of the repressor. It is possible that tryptophan 220 is located near the intersubunit surface, which changes with inducer binding. In this scheme ONPF, which does not affect the chemical shift of tyrosine 282 but does affect tryptophan 220, alters the quaternary structure in a different manner.

The genetic (Miller, 1980), biochemical (Ogata and Gilbert, 1980; Dunaway *et al.*, 1980), and NMR data (Nick *et al.*, 1981), indicate that DNA binding occurs via the NH_2-terminal third of the subunit sequence. Neither ONPF nor IPTG appear to affect the local environments of the NH_2-terminal third of the *lac* repressor as monitored by NMR of fluorotyrosines at residues 7, 12, 17, and 47, as well as tyrosines inserted at 3 and 44 by genetic means (Jarema *et al.*, 1981a). From the information here, combined with the genetic and chemical modification data, it is not possible to locate the inducer or anti-inducer binding sites to a set of specific residues. These approaches can not distinguish direct contact with a ligand from changes in the environment of a residue due to binding of a ligand at a distance. A specific example of this can be seen from our previous data with substituted tyrosines. There is a large upfield shift in the resonance from tyrosine 273 when tyrosine 204 has been substituted with a leucine (Jarema *et al.*, 1981b). At the same time, replacement of the tyrosine at residue 273 with a leucine has a dramatic effect on the inducer-binding property of the repressor (Miller *et al.*, 1979). For the establishment of direct contact one must measure internuclear Overhauser effects or look at the effects

of paramagnetic analogues of inducer and anti-inducer (Rackwitz, 1981a, b) on the resonances under consideration here.

The observations that both allosteric ligands may affect quaternary structure determinants explain the large changes in the overall protein rotational correlation time upon IPTG binding determined by fluorescence measurement (Bandyopadhyay *et al.*, 1981). It appears that these ligands function by altering the inter-subunit interactions in the *lac* repressor and thus the spatial relationships between the DNA binding NH_2-terminal portions. Although no significant changes occur in the radius of gyration determined by neutron diffraction when IPTG binds to the repressor, large changes in the placement of the NH_2-terminal ends could result from the conformational changes we are proposing for the COOH-terminal two-thirds of the repressor and not be reflected in the radius of gyration if the distances from the headpieces to the center of mass remains the same (Charlier *et al.*, 1980). This type of allosteric mechanism we are suggesting has precedent and is understood in detail for hemoglobin (Perutz, 1974).

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