^{19}F Nuclear Magnetic Resonance Studies of Ligand Binding to 3-Fluorotyrosine- and 6-Fluorotryptophan-Containing Dihydrofolate Reductase from *Lactobacillus casei*†

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ABSTRACT: Analogues of *L. casei* dihydrofolate reductase containing either 3-fluorotyrosine or 6-fluorotryptophan have been prepared by growing the organism on media containing the appropriate fluoroamino acid. The ^{19}F nuclear magnetic resonance spectra of these analogues show well-resolved resonances from each of the five tyrosine and five tryptophan residues in the protein, over chemical-shift ranges of 2.66 ppm (fluorotyrosine) and 5.6 ppm (fluorotryptophan). Four of the five fluorotyrosine resonances and all the five fluorotryptophan resonances are affected by the binding of substrate, inhibitors, and/or coenzyme. With the aid of studies of the binding of 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate, which can be considered as "fragments" of the inhibitor methotrexate, it is possible to give a detailed description of the changes in chemical shift on ligand binding. The most notable effects of methotrexate binding are large downfield shifts (1.1–2.7 ppm) of the resonances of one fluorotyrosine and one fluorotryptophan residue; the effect of folate on these resonances is much smaller, suggesting a difference in binding between substrate and inhibitor. The binding of the coenzyme NADPH also leads to large downfield shifts of these same two resonances (one fluorotyrosine and one fluorotryptophan), and in addition a large upfield shift (1.4 ppm) of another fluorotyrosine resonance is observed. The origins of the changes in ^{19}F chemical shift are discussed, and it is concluded that this upfield shift reflects a conformational change produced by coenzyme binding.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduction of dihydrofolate to tetrahydrofolate by NADPH. The enzyme has an essential role in nucleotide synthesis and is the target for the important "anti-folate" drugs such as methotrexate and trimethoprim (Hitchings and Burchall, 1965; Blakley, 1969). As part of a general program aimed at understanding the specificity of inhibitor binding, we have been using nuclear magnetic resonance spectroscopy to study the enzyme and its complexes with substrates and inhibitors.

For such studies it is necessary to resolve resonance signals from individual amino acid residues in the enzyme and to monitor these signals in the presence of substrate analogues. In the proton NMR spectrum of the enzyme we have observed the C2 ^1H signals from the six histidine residues and studied the effects of binding substrate analogues (Birdsall et al., 1977a). In order to examine other aromatic residues we have resorted to isotopic incorporation experiments to allow us to resolve signals for the individual amino acids. For example, a selectively deuterated enzyme containing deuterated phenylalanine, tryptophan, and histidine and [3,5-$^2H_2$]tyrosine gives an ^1H NMR[1] spectrum in which the only signals in the aromatic region are those of the 2,6 protons of the five tyrosine residues, and the effects of ligand binding on individual tyrosines can then be studied in detail (Feeney et al., 1977). Similar studies of the other aromatic amino acids are in progress.

Another approach leading to the resolution of resonances from individual amino acid residues is the incorporation of fluorine-labeled amino acids into the protein (Sykes et al., 1974; Hull and Sykes, 1974, 1975, 1976; Browne and Otvos, 1976; Anderson et al., 1975; Pratt and Ho, 1975). The ^{19}F nucleus is ideal for NMR studies, having a high natural sensitivity (83.3% that of ^1H at constant field) and a very large chemical-shift range. Clearly the only ^{19}F resonances observed will be those of the chosen labeled amino acid, thus solving the resolution problem. However, fluorine substitution is more likely to perturb the properties of the enzyme than selective deuterium substitution, and some caution must be exercised in transferring results from the fluorinated to the normal enzyme.

We report here the preparation and ^{19}F NMR studies of two fluorine-labeled analogues of the dihydrofolate reductase from *Lactobacillus casei*, containing respectively 3-fluorotyrosine and 6-fluorotryptophan.

Materials and Methods

Fluorinated amino acids were obtained from the Sigma Chemical Co. and used without further purification (the racemic forms were employed, since the presence of the D isomer had no deleterious effect on the growth of the organism). Sources of other chemicals are given in previous papers (Dann et al., 1976; Birdsall et al., 1977a; Feeney et al., 1977).

Preparation of Fluorine-Labeled Dihydrofolate Reductase. Liquid cultures of *L. casei* MTX/R were maintained in a fully defined medium containing 1.0 μg/mL methotrexate. The medium was that described by Dann et al. (1976), with the

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[1] Abbreviations used: NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; Gdn·HCl, guanidine hydrochloride; Dnp, dinitrophenyl.

TABLE I: Growth of *L. casei* MTX/R in Media Containing Fluorinated Aromatic Amino Acids and Limiting Concentrations of Fluorinated Amino Acids Which Will Support Growth.

Amino acid	Growth of *L. casei* MTX/R: % of total available L-amino acid	Growth of *L. casei* MTX/R: Growth[a] after 24 h	Limiting concn (% of total available L-amino acid)
4-Fluorotryptophan	67	++	
5-Fluorotryptophan	67	+++	100
6-Fluorotryptophan	67	++	100
3-Fluorotyrosine	90	+++	90

[a] The growth observed with 3-fluorotyrosine represents about 30% of that observed with normal medium; see Table II.

TABLE II: Yield of Fluorinated Enzymes from 200-Liter Fermentations.

Amino acid substituted in medium	Max A_{600}	Total enzyme units	Final pH
Nil	6.8	32 500	5.1[a]
6-Fluorotryptophan 100% of available L-tryptophan	1.25	7 800	5.2
3-Fluorotyrosine 90% of available L-tyrosine	2.1	13 700	5.1[a]

[a] pH maintained at 5.1 to increase cell yield once this value was reached.

casein hydrolysate replaced by a mixture of pure amino acids; for organisms adapted to growth on media containing fluorinated amino acids, the maintenance medium contained the appropriate fluorinated amino acid. The organisms were subcultured weekly and stored in a refrigerator.

The auxotrophy of the organisms was investigated by inoculating 10-mL samples of medium deficient in only one amino acid with 0.2 mL of a washed logarithmic phase culture. The subsequent growth was followed spectrophotometrically at 600 nm and by monitoring the pH (which falls from 6.8 to ca. 4.0 in normal cultures) in conjunction with controls grown on fully defined medium. It was found that *L. casei* MTX/R is unable to synthesize tryptophan, tyrosine, or phenylalanine to a sufficient extent from the nutrients provided in our standard medium to support extensive growth.

The incorporation of amino acids into the dihydrofolate reductase of *L. casei* MTX/R was studied using ^{14}C-labeled tyrosine or tryptophan. Two liters of medium containing the radioactive amino acid tracer was inoculated with 5 mL of a log-phase culture. After incubation at 37 °C for 18 h the cells were harvested by centrifugation, washed three times with 50 mM Tris-Cl buffer solution, pH 7.2, and then disrupted in a Braun homogenizer. After removal of the cell debris, the supernatant solution, about 25 mL, was loaded onto a methotrexate affinity column (Dann et al., 1976) (bed volume 3 mL) and, after thorough washing with 50 mM potassium phosphate–2 M KCl (pH 6.5), was eluted with 50 mM Tris-Cl (pH 8.5) containing 1 M KCl and 2 mM folic acid. The fractions containing enzyme activity were lyophilized. The lyophilized material was resuspended in 5 mL of deionized water and dialyzed against two changes of 6 M Gdn·HCl to denature the protein and remove folic acid. The dialysis bag was then transferred to a vessel containing deionized water. After four changes, the dialysis was discontinued and the protein precipitated with 5% Cl_3CCOOH. The precipitate was collected and washed with diethyl ether, dried, and then hydrolyzed in 6 N HCl for 16 h at 100 °C. HCl was removed and samples were applied to an amino acid analyzer fitted with a stream splitter. Fractions were collected, diluted with a suitable volume of scintillant, and counted in a Packard Tri-Carb liquid scintillation spectrometer. The identity of the amino acids (or, in the case of tryptophan, its hydrolysis products) in the fractions containing above-background counts was established from their position in the eluate, and confirmed by paper chromatography using suitable standards as markers. The results of these experiments showed that there was minimal conversion of one amino acid into another. Thus if the organisms grow on media containing the fluorinated aromatic amino acids, then the incorporation is on a residue-for-residue basis.

Experiments carried out using the commercially available singly fluorinated analogues of tryptophan or tyrosine as the only available source of these amino acids gave little growth except in the case of 5-fluorotryptophan. However, when only 67% of the available L-amino acid was supplied as the fluorinated analogue, all gave reasonable growth after 24 h (Table I).

Where necessary the organisms were adapted to growth on media containing fluorinated amino acids by gradual exposure to an increasing proportion of the aromatic amino acid in its fluorinated form. This process was continued until either total displacement was possible or a limit was reached beyond which no further adaptation occurred. These limiting concentrations are shown in Table I. The cultures grown under these conditions were then maintained for subsequent large-scale fermentation. Once the organisms were established at their limiting concentrations, their auxotrophy for the aromatic amino acids was reinvestigated. In no case was there a change in this property. Selection of the fluorinated amino-acid analogue which would be used in large-scale enzyme preparations was then based on availability and cost.

Two-hundred-liter fermentations of *L. casei* MTX/R using media containing 3-fluorotyrosine and 6-fluorotryptophan were carried out as described elsewhere for the unsubstituted enzyme (Dann et al., 1976).

Even though growth could be supported in media containing fluorinated amino acids, it is clear from Table II that the final cell density and enzyme level in the standard growth medium substantially exceeds those obtained with fluorinated amino acids. The purification and characterization of the fluorinated dihydrofolate reductases was carried out using the methods described previously (Dann et al., 1976) but on reduced scale. The final stage of the purification yielded an approximately 0.2 mM solution of the enzyme in 10 mM potassium phosphate, 100 mM KCl buffer solution, pH 6.5. This solution was lyophilized and stored at −15 °C until required. Solutions for NMR experiments were prepared by dissolving the enzyme in ^2H_2O using one-fifth of the volume from which the enzyme was lyophilized. The enzyme concentration was then 1.0 mM and the salt concentration 50 mM potassium phosphate–500 mM KCl. All solutions were clarified by filtration through Millipore filters before use. The specific activities of the fluorine-labeled enzymes were very similar to that of the normal enzyme, though the labeled enzymes were appreciably less stable.

Samples of the fluorinated enzymes were taken from the material used in NMR experiments and used for tryptic pep-

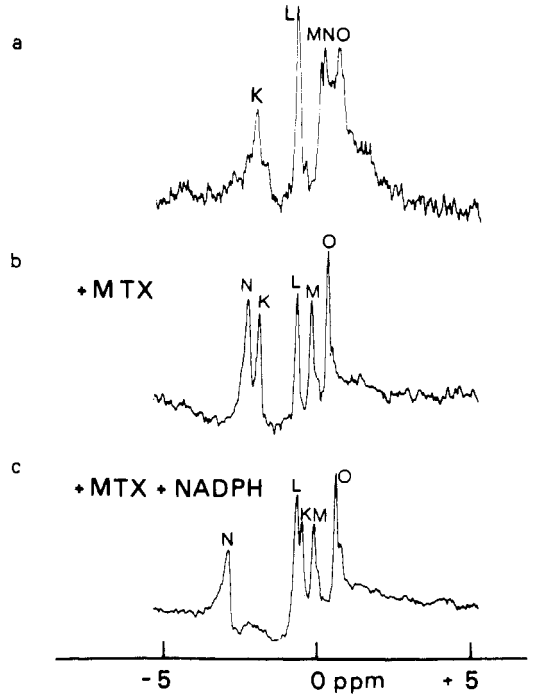

FIGURE 1: ^{19}F NMR spectra of 3-fluorotyrosine-labeled *L. casei* dihydrofolate reductase, alone (a) and in its complexes with methotrexate (b) and methotrexate + NADPH (c). Resonances labeled K, L, etc. arise from residues identified in the text and in Table III as Y_K^F, Y_L^F, etc.

tide maps. Four milligrams of the protein was desalted and precipitated by dialysis against water and then treated with 5% Cl_3CCOOH. The precipitate was washed with diethyl ether and suspended in 50 mM NH_4HCO_3 solution. Trypsin (0.08 mg) (Worthington) was added and the suspension incubated for 2 h at 37 °C. The clear solution obtained was lyophilized and then redissolved in a small volume of 50 mM NH_4HCO_3 and an aliquot containing 20–30 nmol of peptides applied to a sheet of Whatman 3MM chromatography paper. Electrophoresis for 40 min at pH 6.5 (pyridine–acetate buffer solution) and 3 kV in the first dimension was followed by chromatography for 16 h in the second dimension using butanol–acetic acid–water–pyridine (15:3:12:10 v/v) as solvent. Cyanol Blue FF and ϵ-Dnp-lysine were used as markers. The spots were visualized by dipping in ninhydrin–cadmium solution.

The tryptic peptide map obtained from samples of the 6-fluorotryptophan-containing enzyme was identical with that obtained from normal dihydrofolate reductase. The map obtained from the 3-fluorotyrosine-containing enzyme was identical in all respects except that one neutral peptide was not present, and one positively charged peptide appeared which had a somewhat lower R_f value in chromatography than the missing neutral peptide.

Enzyme activity was monitored spectrophotometrically at 340 nm and 25 °C; details are given by Dann et al. (1976).

NMR Measurements. The ^{19}F NMR spectra were recorded at 94.1 MHz using a Varian XL-100 equipped with Fourier transform facilities. The spectra were acquired in the Fourier transform mode using 4096 data points for a 3000-Hz spectral width. Attempts to remove the ^1H–^{19}F spin couplings with a continuous proton-noise-decoupling field resulted in negative nuclear Overhauser effects which completely eliminated the ^{19}F signals. Hull and Sykes (1975) have previously reported similar effects in their ^{19}F NMR investigation of 3-fluorotyrosine-labeled alkaline phosphatase. Such negative Overhauser effects are to be expected for ^{19}F nuclei relaxed by dipolar interactions with protons in proteins where the correlation times are long. By using a procedure where the decoupler was gated *on* during the analytical pulse and the acquisition time but *off* during a delay before the next pulse it was possible to obtain proton-decoupled ^{19}F spectra for the fluorine-labeled enzymes. The spectra used for intensity measurements were usually obtained without proton decoupling and with long delays (8 s) between the pulses.

The enzyme samples were prepared as 1 mM solutions in D_2O (deuterium being used as the field-frequency locking signal) buffer solution containing 50 mM potassium phosphate (pH 6.5) and 500 mM potassium chloride. Sample volumes of 2.0 mL were used in 12-mm sample tubes. The complexes of the enzyme with methotrexate and NADPH were made by adding equimolar quantities of the ligands as solids to the enzyme solutions. 2,4-Diaminopyrimidine and *p*-aminobenzoyl-L-glutamate were added in microliter volumes of 50 or 100 mM solutions of the ligands in D_2O buffer. The pH measurements were made using a Radiometer Model 26 pH meter equipped with a combination glass electrode; the pH values quoted in the text are meter readings, uncorrected for the deuterium isotope effect. The ^{19}F chemical shifts are expressed relative to the sharp signal of the corresponding denatured enzyme. (Some samples which had been stored in solution for a prolonged period showed a small sharp resonance from denatured material.)

Results

3-Fluorotyrosine-Labeled Dihydrofolate Reductase. L. casei dihydrofolate reductase contains five tyrosine residues (Dann et al., 1976; Feeney et al., 1977), and each gives rise to a separate signal in the ^{19}F NMR spectrum of the 3-fluorotyrosine-labeled enzyme (Figure 1a). The chemical shifts of the fluorotyrosine resonances for the enzyme alone and for the various enzyme–ligand complexes are collected in Table III. In the spectrum of the enzyme alone (Figure 1a), the ^{19}F signals appear over a range of 2.66 ppm (compared with a range of 0.3 ppm for the ^1H resonances of these tyrosine residues; Feeney et al., 1977, and unpublished work). The line widths are 15–30 Hz, and three of the signals, designated Y_M^F, Y_N^F, and Y_O^F, are considerably overlapped. These three resonances, together with the sharp resonance of Y_L^F, have chemical shifts within 0.7 ppm of the position of the ^{19}F resonance of denatured 3-fluorotyrosine-labeled dihydrofolate reductase (which may be taken as the position corresponding to a solvent-accessible fluorotyrosine residue). The resonance of Y_K^F, in contrast, is 2.07 ppm to low field of the signal from the denatured protein.

Addition of methotrexate leads to a marked sharpening of all the resonances, and changes in chemical shift of three of the five signals (Figure 1b). Since methotrexate binds very tightly to the enzyme ($K_a > 10^8$ M^{-1}; Dann et al., 1976), exchange between bound and free states is slow on the NMR time scale and we cannot directly connect the resonances in the enzyme spectrum with those in the spectrum of the enzyme–methotrexate complex. As in previous work (Birdsall et al., 1977a; Feeney et al., 1977), we have used weakly binding "fragments" of methotrexate such as 2,4-diaminopyrimidine ($K_a \sim 10^4$ M^{-1}) which are much more likely to be in rapid exchange, as an aid in assigning the spectrum of the methotrexate complex.

Figure 2 shows the ^{19}F spectra of 3-fluorotyrosine-labeled dihydrofolate reductase in the presence of increasing concentrations of 2,4-diaminopyrimidine. Three of the five resonances show changes in chemical shift on addition of 2,4-diami-

TABLE III: The ^{19}F Chemical Shifts[a] of the 3-Fluorotyrosine Resonances of 3-Fluorotyrosine-Labeled Dihydrofolate Reductase and Its Complexes with Ligands.

Ligand	Y_K^F	Y_L^F	Y_M^F	Y_N^F	Y_O^F
None	2.07	0.68	−0.17[b]	−0.38[b]	−0.59
Folate	1.91	0.68	0.16	0.68	−0.55
Methotrexate	1.94	0.68	0.20	2.30	−0.35
2,4-Diaminopyrimidine	1.99	0.64	0.20	1.59	−0.28
2,4-Diaminopyrimidine + *p*-aminobenzoyl-L-glutamate	1.97	0.68	0.18	1.83	−0.36
NADPH	0.64 (±0.5)	0.64 (±0.5)	0.64 (±0.5)	0.64 (±0.5)	−0.89
NADPH + *p*-aminobenzoyl-L-glutamate	0.64 (±0.5)	0.64 (±0.5)	0.64 (±0.5)	0.64 (±0.5)	−0.89
NADPH + 2,4-diaminopyrimidine + *p*-aminobenzoyl-L-glutamate	0.72	0.72	0.72	2.56	−0.60
NADPH + methotrexate	0.52	0.68	0.12	2.94	−0.61

[a] Measured in ppm (±0.05 ppm) from the ^{19}F signal of 3-fluorotyrosine in denatured enzyme; positive shifts to low field. See text for comments on assignments. [b] ±0.10 ppm.

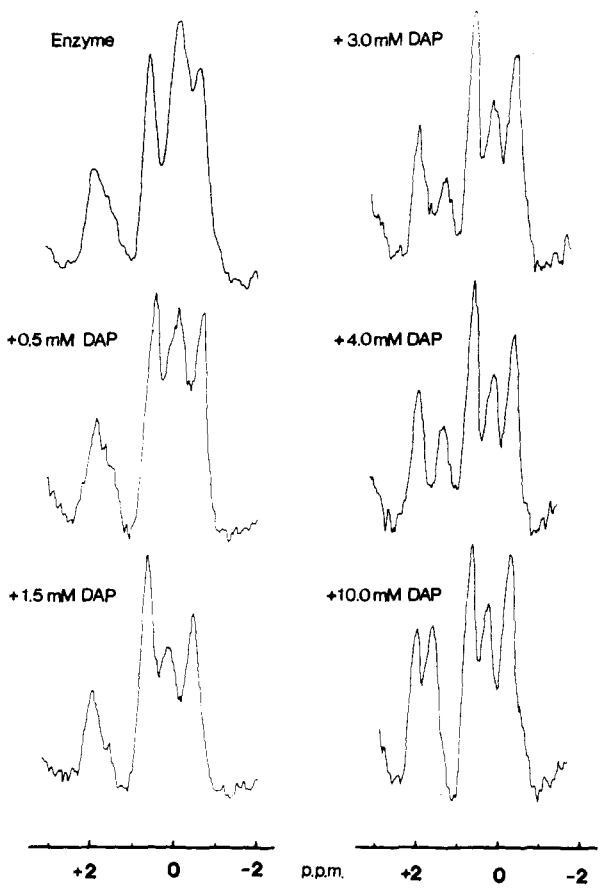

FIGURE 2: ^{19}F NMR spectra of 3-fluorotyrosine-labeled *L. casei* dihydrofolate reductase, alone and in the presence of various concentrations of 2,4-diaminopyrimidine. These spectra were obtained *without* proton irradiation.

nopyrimidine, those of tyrosines Y_K^F and Y_L^F being unaffected. The resonances of Y_M^F and Y_O^F show progressive changes in chemical shift on addition of increasing amounts of 2,4-diaminopyrimidine, behavior characteristic of rapid exchange between the free enzyme and the complex. These resonances shift downfield, by 0.37 and 0.31 ppm, respectively. The observation of rapid exchange between sites differing in chemical shift by 35 Hz sets an upper limit to the lifetime of the complex, $\tau \lesssim 4.6$ ms (corresponding to a lower limit to the dissociation rate constant, $k_{off} \gtrsim 220\ s^{-1}$). The resonance of Y_N^F decreases in intensity on addition of 2,4-diaminopyrimidine, and a new resonance, corresponding to Y_N^F in the complex, appears about 1.6 ppm downfield. This "new" resonance increases in intensity, sharpens, and shifts slightly further downfield as the concentration of 2,4-diaminopyrimidine is further increased. The behavior of the resonance of Y_N^F is thus characteristic of slow exchange, though the observation that the resonance corresponding to the complex does shift slightly with increasing 2,4-diaminopyrimidine concentration indicates that this resonance must be approaching intermediate exchange under these conditions. Overlap with other resonances prevents a detailed line shape analysis, but we can set approximate limits to the lifetime, $4/2\pi\Delta\nu \gtrsim \tau \gtrsim \sqrt{2}/2\pi\Delta\nu$, where $\Delta\nu$ is the chemical-shift separation for the resonance of Y_N^F, between the free enzyme and the complex. For $\Delta\nu = 186$ Hz, 3.4 ms $\gtrsim \tau \gtrsim$ 1.2 ms, or 295 $s^{-1} \lesssim k_{off} \lesssim 833\ s^{-1}$ (consistent with the lower limit for k_{off} obtained above).

Thus the binding of 2,4-diaminopyrimidine causes downfield shifts of the resonances of three tyrosine residues, Y_M^F (by 0.37 ppm), Y_N^F (by 1.97 ppm), and Y_O^F (by 0.31 ppm) (see also Table III). Addition of *p*-aminobenzoyl-L-glutamate to form the ternary enzyme-2,4-diaminopyrimidine-*p*-aminobenzoyl-L-glutamate complex affects essentially only one tyrosine residue, the resonance of Y_N^F being shifted 0.24 ppm further downfield to 1.83 ppm (Table III); any effects on the other resonances are less than 0.1 ppm. We can now use these results to assign the resonances in the spectrum of the enzyme-methotrexate complex (Figure 1b). In this spectrum, the resonances at 1.94 and 0.68 ppm are very close to the positions of the resonances of Y_K^F and Y_L^F in the free enzyme and in the complexes with the "fragments"; these two tyrosine residues are apparently unaffected by methotrexate binding. Similarly, the resonances at 0.20 and −0.35 ppm in the spectrum of the enzyme-methotrexate complex are within 0.02 ppm of the positions of the resonances of Y_M^F and Y_O^F, respectively, in the enzyme-2,4-diaminopyrimidine-*p*-aminobenzoyl-L-glutamate complex, and we can assign them with some confidence. The remaining resonance, at 2.30 ppm, in the spectrum of the enzyme-methotrexate complex must thus be that of Y_N^F, which has been shifted 2.68 ppm downfield on methotrexate binding. This is almost 0.5 ppm greater than the shift of this resonance on forming the ternary enzyme-2,4-diaminopyrimidine-*p*-aminobenzoyl-L-glutamate complex. An alternative assignment of the resonances in the spectrum of the enzyme-

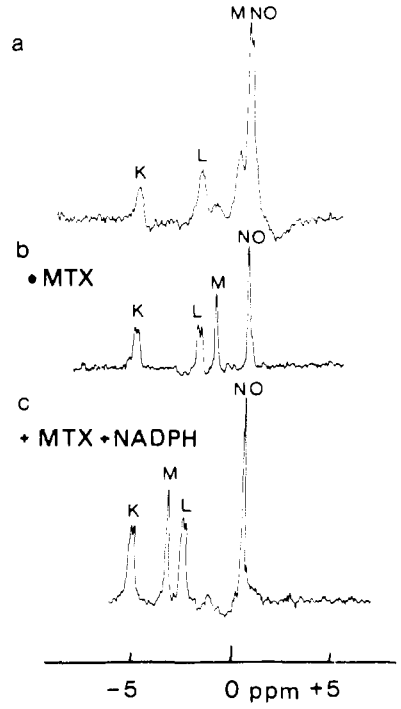

FIGURE 3: ^{19}F spectra of 6-fluorotryptophan-labeled *L. casei* dihydrofolate reductase, alone (a) and in its complexes with methotrexate (b) and methotrexate + NADPH (c). Resonances labeled K, L, etc., here and in Figure 4 arise from residues identified in the text and Table IV as W_K^F, etc.

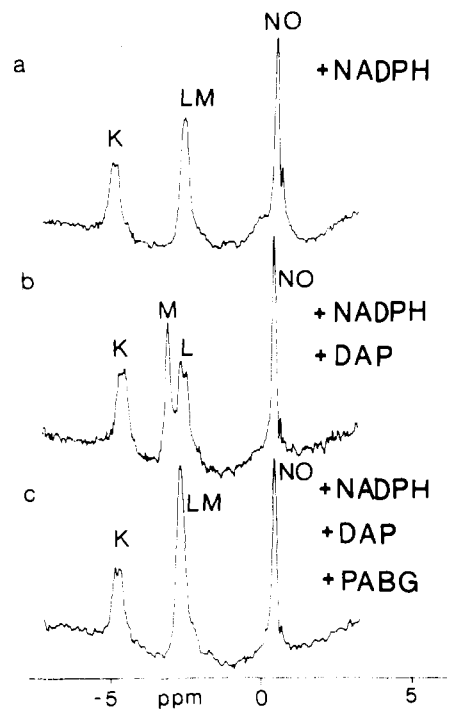

FIGURE 4: ^{19}F NMR spectra of 6-fluorotryptophan-labeled *L. casei* dihydrofolate reductase in its complex with NADPH (a), and after addition of saturating concentrations of 2,4-diaminopyrimidine (b), followed by *p*-aminobenzoyl-L-glutamate (c).

methotrexate complex would be to assign the resonance at 1.94 ppm to Y_N^F (in better agreement with its position in the "fragment" complex) and that at 2.30 ppm to Y_K^F. This would imply that tyrosine Y_K^F, which is hardly affected by 2,4-diaminopyrimidine or *p*-aminobenzoyl-L-glutamate, is significantly affected by methotrexate; this seems unlikely (see Discussion) but cannot be completely ruled out on the available evidence.

The effects of folate on the spectrum of 3-fluorotyrosine-labeled dihydrofolate reductase (Table III) are quite distinct from those of methotrexate. Again tyrosines Y_K^F and Y_L^F are essentially unaffected, but the downfield shift of the resonance of Y_O^F observed with methotrexate is not seen with folate. Folate binding, like methotrexate binding, leads to a downfield shift of the resonances of Y_M^F and Y_N^F; on the most probable assignment, the shift of Y_M^F is similar to that produced by methotrexate (0.33 vs. 0.37 ppm) but that of Y_N^F is much less (1.06 vs. 2.68 ppm).

The spectrum of the enzyme-NADPH complex shows only two resonances: an intense broad one at 0.64 ppm, and a small sharper one at −0.89 ppm. The position of the resonance at 0.64 ppm is consistent with a lack of effect of NADPH binding on tyrosine Y_L^F, but all other four resonances must have been shifted—those of Y_K^F and Y_N^F by 1.0-1.4 ppm. The most straightforward assignment (Table III) is that the resonance at −0.89 ppm is that of Y_O^F, with the other four resonances appearing at ca. 0.64 ppm; some support for this assignment comes from a study of the effects of adding the "fragments" of methotrexate to the enzyme-NADPH complex. *p*-Aminobenzoyl-L-glutamate has no effect (Table III) but subsequent addition of 2,4-diaminopyrimidine to give the quaternary complex enzyme-NADPH-2,4-diaminopyrimidine-*p*-aminobenzoyl-L-glutamate affects two of the ^{19}F resonances. The signal at −0.89 ppm is shifted downfield by 0.29 ppm, while one of the resonances at ~0.64 ppm shifts downfield by ca. 1.9 ppm to 2.56 ppm. The "center of gravity" of the remaining resonance shifts slightly to 0.72 ppm. A comparison of these effects with the corresponding ones in the absence of NADPH clearly suggests that the two resonances affected are those of Y_O^F (downfield shift of 0.29 ppm vs. 0.23 ppm) and Y_N^F (downfield shift of 1.9 ppm vs. 2.2 ppm).

The ^{19}F spectrum of the ternary enzyme-NADPH-methotrexate complex (Figure 1c) can now be at least partially assigned. The resonances at 0.68 and −0.61 ppm must be those of Y_L^F and Y_O^F, respectively. The resonance at 2.94 ppm is probably that of Y_N^F (appearing at 2.56 ppm in the enzyme-NADPH-2,4-diaminopyrimidine-*p*-aminobenzoyl-L-glutamate complex and at 2.30 ppm in the enzyme-methotrexate complex). The remaining resonances, at 0.52 and 0.12 ppm corresponding to Y_K^F and Y_M^F, cannot be assigned with confidence.

6-Fluorotryptophan-Labeled Dihydrofolate Reductase. There are five tryptophan residues in *L. casei* dihydrofolate reductase (Dann et al., 1976); the ^{19}F NMR spectrum of the 6-fluorotryptophan-labeled enzyme is shown in Figure 3a, and the chemical shifts of the ^{19}F resonances in the enzyme and its complexes are listed in Table IV. The ^{19}F resonances in the spectrum of the enzyme alone appear over a range of 5.6 ppm. Three resonances, designated W_M^F, W_N^F, and W_O^F are 0.5-1.0 ppm to high field of the resonance position of 6-fluorotryptophan itself, while the other two, W_L^F and W_K^F, are 1.34 and 4.52 ppm, respectively, to low field of the resonance position of the denatured enzyme.

As was observed for the 3-fluorotyrosine-labeled enzyme (Figure 1), the fluorotryptophan resonances in the spectrum of the enzyme alone are relatively broad (~20 Hz), those of W_K^F and W_L^F being somewhat broader than the others. Addition of methotrexate (Figure 3b) or NADPH (Figure 4a) leads to a marked sharpening of the resonances and reveals that the resonances of W_K^F and W_L^F are "doublets", with a split-

TABLE IV: The ^{19}F Chemical Shifts[a] of the 6-Fluorotryptophan Resonances of 6-Fluorotryptophan-Labeled Dihydrofolate Reductase and Its Complexes with Ligands.

Ligand	W_K^F	W_L^F	W_M^F	$W_N^F + W_O^F$
None	4.52	1.34	−0.51	−1.08
Folate	4.30	1.51	−0.22	−1.05
Methotrexate	4.80	1.47	0.62	−1.03
Methotrexate + NADPH	4.72	2.08	2.96	−0.97
$NADP^+$	4.65	1.59	−0.39	−1.02
NADPH	4.61	2.21	2.21	−0.86
NADPH + 2,4-diaminopyrimidine	4.39	2.31	2.81	−0.77
NADPH + 2,4-diaminopyrimidine + *p*-aminobenzoyl-L-glutamate	4.59	2.46	2.46	−0.73

[a] In ppm (±0.05 ppm) from the ^{19}F resonance of 6-fluorotryptophan; positive shifts to low field. See text for comments on assignments.

ting of approximately 17 Hz (0.18 ppm). The spectra shown in these Figures were obtained under conditions of proton noise decoupling, so that these splittings do not represent ^1H-^{19}F spin-spin coupling; their possible origins are discussed below.

The resonances in the spectrum of the enzyme-methotrexate complex (Figure 3b) can be assigned in a fairly straightforward manner. Comparison of the spectra in Figures 3a and 3b shows that the resonances of residues W_N^F and W_O^F have not been affected by methotrexate binding, while that of W_M^F has been shifted downfield. The lowest field resonance can be taken to be that of W_K^F, which undergoes a modest downfield shift on methotrexate binding (0.28 ppm); any other assignment for this resonance would require us to postulate very large (>3 ppm) shifts in opposite directions for *two* resonances. The two resonances in the center of the spectrum must then arise from W_L^F and W_M^F; the one to lower field is close (0.13 ppm downfield) to that of W_L^F in the spectrum of the enzyme alone and is assigned to W_L^F. The assignments of the resonances of W_K^F and W_L^F are also supported by the doublet character of these resonances (although the doublets cannot be resolved in the spectrum of the enzyme alone (Figure 3a), the resonances of W_K^F and W_L^F are significantly broader than the other resonances in this spectrum).

Thus the binding of methotrexate leads to a large downfield shift (1.13 ppm) of the resonance of fluorotryptophan W_M^F, and smaller downfield shifts of the resonances of two other fluorotryptophans. In contrast, addition of folate, which affects the same three resonances (Table IV), has a much smaller effect on W_M^F, shifting its resonance only 0.29 ppm downfield, and leads to an *upfield* shift of the resonance of W_K^F.

The binding of NADPH (Figure 4a) also leads to a large (2.72 ppm) downfield shift of the resonance of W_M^F, so that it coincides with that of W_L^F, the latter resonance having been shifted 0.87 ppm downfield. The remaining three resonances are affected to a much lesser extent (downfield shifts of ≤0.22 ppm), though NADPH binding clearly has a greater effect on the resonance of $W_N^F + W_O^F$ than does the binding of substrate analogues. Addition of 1 molar equiv of $NADP^+$ (Table IV) has only relatively small effects on the spectrum (<0.25 ppm); in particular, the large downfield shift of the resonance of W_M^F produced by NADPH is not seen with $NADP^+$.

Progressive addition of the methotrexate "fragment" 2,4-diaminopyrimidine to the enzyme-NADPH complex leads to a number of further changes in the spectrum (Figure 4b and Table IV). The resonance of residue W_K^F is shifted upfield by 0.22 ppm, while those of residues W_L^F and W_M^F (distinguished from one another again by the doublet character of W_L^F) are shifted downfield by 0.10 and 0.60 ppm, respectively. Subsequent addition of *p*-aminobenzoyl-L-glutamate (Figure 4c) to form the quaternary complex reverses some of the effects of 2,4-diaminopyrimidine. Thus, the resonance of W_K^F is shifted downfield to the same position it occupied in the spectrum of the enzyme-NADPH complex, while that of W_M^F is shifted upfield. The resonance of W_L^F, however, is shifted further downfield, so that once again the resonances of W_L^F and W_M^F are coincident.

The spectrum of the enzyme-methotrexate-NADPH complex (Figure 3c) shows only a general resemblance to that of the quaternary complex with the "fragments" of methotrexate (Figure 4c). It is clear that in both complexes the resonance of W_M^F is substantially (≥3 ppm) further downfield than in the free enzyme, but its position in the two complexes differs by 0.5 ppm. Differences of 0.1–0.4 ppm between the two complexes are seen in the positions of the other resonances (Table IV). (It should be noted that in making the resonance assignments in these spectra it has been assumed that the resonances appearing as doublets are always those of W_K^F and W_L^F).

Discussion

It is well known that the shielding of a fluorine nucleus is much more sensitive to changes in its environment than is that of a proton. Thus it is not surprising that both the chemical differences between different fluorotyrosine or fluorotryptophan residues in native dihydrofolate reductase and the changes in chemical shift produced by ligand binding are up to an order of magnitude larger than, for example, those of the 2,6 protons (Feeney et al., 1977) or the 3,5 protons (B. Birdsall, J. Feeney, and G. C. K. Roberts, unpublished work) of the tyrosine residues.

The Origins of the ^{19}F Shielding Effects. Neighbor anisotropic shielding contributions, such as ring current effects, are expected to be similar for fluorine and protons. An approximate upper limit to their contribution in the case of 3-fluorotyrosine can thus be set by the observed chemical-shift differences of the 3,5 protons of the tyrosine residues in the selectively deuterated enzyme and its complexes. The chemical-shift range of the five tyrosine 3,5-proton resonances of dihydrofolate reductase is 0.28 ppm, and the largest chemical shift change on ligand binding is 0.25 ppm (B. Birdsall, J. Feeney, and G. C. K. Roberts, unpublished work). The corresponding values for the ^{19}F resonances of the 3-fluorotyrosine-labeled enzyme are 2.66 ppm and 3.32 ppm. It is clear that, if there is no conformational difference between the normal and fluorine-labeled enzymes, the contribution of neighbor anisotropic effects to the ^{19}F chemical-shift differences can be ignored.

The most likely origin of these through-space shielding effects on the ^{19}F nuclei is an electric-field effect (Boden et al., 1964; Feeney et al., 1966). The electric field contribution to shielding can be expressed (Buckingham, 1960; Buckingham et al., 1963) as:

$$\delta_{EF} = -A\Delta E_z - B(\Delta E^2 + \Delta\langle E^2\rangle)$$

where ΔE_z is the change in the component of the electric field (arising from charged or polar groups in the environment) along the C–F bond, ΔE^2 is the change in the square of the electric fields at the fluorine nucleus, and $\Delta\langle E^2\rangle$ is the change in the time-averaged square of the electric fields at the fluorine nucleus arising from fluctuating bond dipoles; A and B are constants. It has been shown (Feeney et al., 1966) that the second-order electric field effect dominates the shielding. Second-order electric field effects lead to deshielding contributions (downfield shifts); the van der Waals contribution ($\Delta\langle E^2\rangle$) to the second-order term, which arises from London dispersion forces, has a r^{-6} distance dependence, and the deshielding contribution can be large when the interacting group is close to the nucleus (>10 ppm at 2.5 Å, falling to <1 ppm at 5 Å). Hull and Sykes (1976) have demonstrated an approximate correlation between the chemical shifts of the ^{19}F resonances of 3-fluorotyrosine-labeled alkaline phosphatase and $\Sigma_i r_i^{-6}$ (where r_i's are the distances to neighboring protons) estimated from relaxation measurements.

Since the second-order electric field contribution leads to downfield shifts, one would in general expect the resonances of "buried" fluoroamino acids in proteins to appear to low field of those of the corresponding group in a solvent-accessible environment. This seems to be true for the 3-fluorotyrosine (Sykes et al., 1974) and 4-fluorotryptophan (Browne and Otvos, 1976) resonances of labeled alkaline phosphatase. In the labeled dihydrofolate reductases, two of the fluorotyrosine and one of the fluorotryptophan residues (Y_M^F, Y_N^F, and W_M^F) give rise to resonances within about 0.5 ppm of the resonance position of the denatured protein, and can be regarded as solvent accessible. Three residues (tyrosine Y_O^F and tryptophans W_N^F and W_O^F) give resonances to high field of the "solvent accessible" position, but only by 0.6–1.0 ppm, while four have resonances to low field, by as much as 4.5 ppm.

In considering the origin of a change in chemical shift of a resonance from the protein resulting from ligand binding, one is always faced with the problem of distinguishing between direct and indirect effects. Does the change in chemical shift of a resonance from a particular residue reflect the proximity of the bound ligand to this residue, or does it reflect a conformational change which alters the environment of residues remote from the binding site? If the chemical-shift change arises from an additional second-order electric field contribution from atoms or groups on the ligand (which must therefore be very close to the ^{19}F nucleus being observed), a downfield shift will result. On the other hand, a conformational change can result in either a downfield or an upfield shift, depending on whether the interacting groups are moving closer to or further away from the observed nucleus. Thus, for second-order electric field shifts, an upfield shift on ligand binding can only result from a ligand-induced conformational change; this criterion must clearly only be applied to shifts which are sufficiently large for any contributions from, e.g., neighbor anisotropic effects to be neglected. In fact, as will be discussed below, the majority of the large ligand-induced shifts are to low field, though there is one example of a high-field shift.

Line-Broadening and Splitting Effects. The broad lines observed in the spectra of the fluorotyrosine- or fluorotryptophan-labeled enzymes are markedly sharpened on the addition of ligands (whether substrates, substrate analogues, or coenzymes). Similar, though rather less pronounced, line sharpening on ligand binding is observed for the proton resonances of the histidine (Birdsall et al., 1977a) and tyrosine (Feeney et al., 1977) residues. If these were simply dipolar relaxation effects, one would have to propose that ligand binding decreased the correlation time of all these residues, and/or increased their distance from neighboring residues (since for all these resonances a major contribution to the dipolar relaxation is likely to come from protons of other residues; cf. Hull and Sykes, 1975). This would imply a rather widespread loosening of the protein structure on ligand binding, which seems most unlikely, particularly since ligand binding to dihydrofolate reductase is accompanied by a dramatic increase in thermal stability.

A more probable explanation for the broad lines observed in the spectra of the enzyme alone is that they result from the exchange of the enzyme between a number of different conformational forms. The sharpening of the resonances on ligand binding could then occur if the complex existed in only a single conformation. There is clear evidence from stopped-flow studies of the kinetics of coenzyme and inhibitor binding (R. W. King, S. Dunn, and J. G. Batchelor, unpublished work) that *L. casei* dihydrofolate reductase exists in at least two interconvertible forms, one of which preferentially binds ligands.

The line widths observed in the complexes are in the range expected for dipolar relaxation, supporting the predicted absence of chemical-shift anisotropy relaxation in a protein of this size (Hull and Sykes, 1975).

Some contribution to the line width of the resonances of fluorotryptophans W_K^F and W_L^F probably arises from a splitting of these resonances. With the narrower lines seen on addition of methotrexate or NADPH, these two resonances are clearly seen as "doublets" with a separation of about 17 Hz; this does not represent an ^1H–^{19}F spin–spin coupling. The two remaining possibilities are the following. (a) The "splitting" is a chemical-shift difference, reflecting the coexistence of approximately equal amounts of *either* two chemically different forms of the enzyme, *or* two conformationally different forms which are not rapidly interconverting; in either case the two forms would have to differ only locally around these two fluorotryptophan residues. Alternatively, (b) the "splitting" represents a through-space ^{19}F–^{19}F spin–spin coupling, and fluorotryptophans W_K^F and W_L^F are close together in space. Tentative arguments for and against each of these possibilities can be advanced; a definitive decision between them must await variable field and triple resonance experiments currently in progress.

Effects of Ligand Binding: 3-Fluorotyrosine-Labeled Enzyme. One of the five fluorotyrosine residues, Y_L^F, shows no change in its resonance position on addition of any of the ligands yet studied. Since ^{19}F chemical shifts are hypersensitive to changes in the environment of the nucleus, one can be confident that the absence of a chemical-shift change corresponds to the absence of a change in environment. This resonance appears 0.68 ppm to low field of that of the denatured protein, so that it is clearly not in a solvent-like environment; it must be in a region of the protein remote from the binding site whose conformation is unaffected by ligand binding.

All the other four fluorotyrosine residues are affected to a greater or lesser extent by the binding of both substrate analogues and coenzyme. Considering first the changes observed

on forming the binary complexes with the substrate, folate, or substrate analogues, by far the largest effect is that on the resonance of fluorotyrosine Y_N^F. This resonance undergoes a downfield shift of 1.06–2.68 ppm when the substrate site is occupied; for the other three resonances, the shifts are ≤0.37 ppm. This large downfield shift of the resonance of Y_N^F is the kind of effect one would expect for a residue directly affected by ligand binding but a conformational effect cannot be excluded. The chemical shift of this resonance in the enzyme–2,4-diaminopyrimidine–*p*-aminobenzoyl-L-glutamate complex is 1.83 ppm, compared with 2.30 ppm in the enzyme–methotrexate complex. A significant difference between these complexes is indicated, but clearly the "fragments" are able to produce broadly the same kind of shift as methotrexate itself. The major part of the change in chemical shift produced by the binding of both fragments is seen when only 2,4-diaminopyrimidine is bound (2.21 and 1.97 ppm, respectively), suggesting that it is the diaminopteridine ring portion of methotrexate which is responsible for the effect on fluorotyrosine Y_N^F. In this connection it is interesting that folate, an oxyaminopteridine, has a very much smaller effect than does methotrexate (1.06 vs. 2.68 ppm) on this resonance. Of the remaining fluorotyrosines, Y_M^F and Y_K^F are affected to very much the same extent by folate and methotrexate, but folate has essentially no effect on Y_O^F, while methotrexate produces a downfield shift of 0.24 ppm. The magnitude of this shift is such that it cannot be interpreted with any confidence, but may reflect a conformational difference between the folate and methotrexate complexes, for which there is other evidence (Birdsall et al., 1977a; Feeney et al., 1977; B. Birdsall, B. Kimber, J. Feeney, and G. C. K. Roberts, unpublished work).

The binding of the coenzyme, NADPH, affects the chemical shifts of all the four fluorotyrosine resonances which are affected by substrate or inhibitor binding. The resonances of Y_M^F and Y_N^F undergo moderately large downfield shifts, of ca. 0.8[2] and 1.02 ppm, respectively. The change in chemical shift of the resonance of Y_N^F on forming the ternary enzyme–NADPH–methotrexate complex (3.32 ppm) is approximately the sum of the effects of methotrexate (2.68 ppm) and NADPH (1.02 ppm); such an additivity of chemical-shift effects is consistent with (but cannot prove) the suggestion that ligand effects on this resonance are direct ones, due to the proximity of the bound ligands (both methotrexate and NADPH) to fluorotyrosine Y_N^F. As for the binary complexes, the effect of methotrexate on the resonance of Y_N^F is primarily due to its pteridine ring, as indicated by the lack of effect of *p*-aminobenzoyl-L-glutamate and the large downfield shift produced by 2,4-diaminopyrimidine.

In contrast to the large downfield shifts of the resonances of Y_M^F and Y_N^F, the binding of NADPH leads to an *upfield* shift of 1.43 ppm of the resonance of Y_K^F. As noted above, second-order electric field shifts are downfield, so that this large change in chemical shift cannot be due to a direct effect of the bound ligand, but must reflect a conformational change produced by NADPH binding. The nature of this change must be such as to move charged or polar groups *away* from the fluorine nucleus—though the r^{-6} distance dependence of the van der Waals shielding contribution means that this movement need not be more than a few tenths of an angstrom. The effect of methotrexate binding on the resonance of Y_K^F is less than a tenth that of NADPH, so that this fluorotyrosine is apparently in a region of the protein molecule whose conformation is altered by coenzyme binding but only marginally by inhibitor or substrate binding.

It is of interest to compare the effects of ligand binding on the ^{19}F resonances of the fluorotyrosine residues with those on the proton resonances. By preparing selectively deuterated enzyme, we have been able to study in detail both the 2,6 protons (Feeney et al., 1977) and the 3,5 protons (B. Birdsall, J. Feeney, and G. C. K. Roberts, unpublished work) of the tyrosine residues of dihydrofolate reductase. As noted above, both the chemical-shift range and the magnitude of the ligand-induced shifts are almost an order of magnitude less for the proton resonances than for the fluorine resonances. This has repercussions in the analysis of the effects of ligand binding; thus, for example, while methotrexate binding affects four and folate binding three of the fluorotyrosine resonances, only three and one, respectively, of the proton resonances are shifted by an amount greater than the error in measuring line positions. It is apparent that fluorine shifts are more sensitive to small perturbations in the environment of individual residues than are proton chemical shifts. A detailed comparison of all the available information on the tyrosine residues must await experiments in progress to connect the 2,6- and 3,5-proton resonances of individual tyrosine residues.

Effects of Lignad Binding: 6-Fluorotryptophan-Labeled Enzyme. All the five fluorotryptophan resonances are affected by ligand binding, though the effects of substrate or inhibitor binding on W_N^F and W_O^F, and those of coenzyme binding on W_K^F are minimal. As was found for the fluorotyrosine residues, the major effect of forming the binary methotrexate complex is a large downfield shift (1.13 ppm) of one resonance, that of W_M^F, the other resonances being affected by ≤0.28 ppm. Once again, folate has a much smaller effect on this resonance (W_M^F) than does methotrexate. Interestingly, the binding of folate produces an *upfield* shift of the resonance of W_K^F, while methotrexate produces a *downfield* shift of comparable magnitude (0.22–0.28 ppm); the size of the shift is not sufficient for it to be ascribed with confidence to a second-order electric field shift, so that the upfield shift produced by folate binding cannot be interpreted as a conformational rather than a direct effect.

The oxidized and reduced forms of the coenzyme have quite different effects on the spectrum. The difference is particularly striking for residue W_M^F, whose resonance is shifted downfield 2.72 ppm by NADPH but only 0.11 ppm by $NADP^+$. It has been shown by ^{31}P and ^1H NMR (Feeney et al., 1975, 1977; Birdsall et al., 1977b) that the adenine, 2′-phosphate, and pyrophosphate groups of the coenzyme bind in a very similar way in both $NADP^+$ and NADPH; the difference between them must be localized to the region of the nicotinamide ring. If the large downfield shift of the resonance of W_M^F produced by NADPH is a direct effect, then this fluorotryptophan residue must be close to the nicotinamide ring of NADPH, but not to that of $NADP^+$. The effects of methotrexate and NADPH on this resonance are somewhat less than additive, and the experiments with the "fragments" show that its shift is influenced by both the 2,4-diaminopyrimidine and the *p*-aminobenzoyl-L-glutamate moieties. A single tryptophan residue has been implicated in the activity of dihydrofolate reductase by chemical modification studies of the enzyme from *E. coli* (Williams, 1975), *S. faecium* (Warwick et al., 1972), and from two strains of *L. casei* (Liu and Dunlap, 1974; K. Hood and G. C. K. Roberts, unpublished work), and we have shown that modification of this tryptophan residue leads to a

[2] Problems of signal overlap and assignment make it difficult to describe the behavior of the resonance of Y_M^F precisely in complexes containing NADPH.

dramatic (greater than 1000-fold) reduction in the affinity of the enzyme for NADPH (K. Hood and G. C. K. Roberts, unpublished work). It is tempting to suggest that this tryptophan residue corresponds to the fluorotryptophan W_M^F, but definitive conclusions must await NMR studies of the chemically modified enzyme. If fluorotryptophan W_M^F is directly involved in the binding of NADPH, then it is possible that the fluorine substitution will affect the precise mode of binding of the coenzyme, and some caution must be exercised in transferring the present results to the normal enzyme. Although the specific activity of the fluorotryptophan-labeled enzyme is very similar to that of the normal enzyme, the binding constant of NADPH has not been determined. Studies of the proton resonances of the tryptophan residues, using selectively deuterated dihydrofolate reductase, are in progress.

Conclusions

We have shown that both 3-fluorotyrosine and 6-fluorotryptophan can be incorporated biosynthetically into *L. casei* dihydrofolate reductase to yield active enzyme. The ^{19}F NMR spectra of these fluorine-labeled enzymes are well resolved, and show that at least two of the fluorotyrosine and two of the fluorotryptophan residues are in solvent-inaccessible environments. Ligand binding affects four of the five fluorotyrosine and all five fluorotryptophan resonances. One fluorotyrosine and one fluorotryptophan residue, which are in solvent-like environments in the native enzyme, show large downfield shifts of their ^{19}F resonances on the binding of methotrexate or NADPH. These effects are consistent with either a direct effect of the ligands on these residues, or an effect mediated by a conformational change. In both cases, the effects of folate, a substrate, are substantially less than those of the inhibitor methotrexate, indicating a significant difference in their mode of binding. The binding of NADPH causes a large upfield shift of one fluorotyrosine resonance; this is due to a conformational change accompanying coenzyme binding. Substrate and inhibitor binding has little effect on this resonance.

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