^{19}F Nuclear Magnetic Resonance Chemical Shifts of Fluorine Containing Aliphatic Amino Acids in Proteins: Studies on *Lactobacillus casei* Dihydrofolate Reductase Containing (2*S*,4*S*)-5-Fluoroleucine[||]

James Feeney,*,† John E. McCormick,† Christopher J. Bauer,† Berry Birdsall,† Claire M. Moody,‡ Bernard A. Starkmann,‡ Douglas W. Young,‡ Peter Francis,§ Robert H. Havlin,⊥ William D. Arnold,⊥ and Eric Oldfield⊥

Contribution from the Molecular Structure Division and Biomedical NMR Centre, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK, School of Chemistry and Molecular Sciences, University of Sussex, Falmer, Brighton, BN1 9QJ, UK, The Wellcome Research Laboratories, Langley Court, South Eden Park Road, Beckenham, Kent, BR3 3BS, UK, and Department of Chemistry, University of Illinois at Urbana-Champaign, 505 South Mathews Avenue, Urbana, Illinois 61801

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Abstract: We have prepared *Lactobacillus casei* dihydrofolate reductase containing biosynthetically incorporated (2*S*,4*S*)-5-fluoroleucine ([5-F]-Leu DHFR) and have obtained its ^{1}H and ^{19}F NMR spectra at 9.4 Tesla. The ^{19}F spectrum of [5-F]-Leu DHFR showed 12 fairly sharp peaks (one containing two overlapped signals) for the 13 leucine residues in DHFR, covering a chemical shift range of 15 ppm. The large range of chemical shifts observed could not be explained solely in terms of the electrostatic field effects due to local charge fields and is thought to have a second contribution from side-chain conformational differences (γ-gauche effects) between different leucine residues, making ^{19}F NMR of aliphatic amino acids in proteins a potentially useful new probe of protein structure.

Introduction

Over the past 25 years there have been numerous reports of ^{19}F NMR studies on ^{19}F-labeled proteins. Most of these investigations have concerned proteins with fluorine-containing aromatic amino acids[1−16] and have taken advantage of the sensitivity of ^{19}F chemical shifts to changes in the local environment to follow structural changes under different sample conditions. In earlier studies we introduced [3-F]-Tyr, [3-F]-Phe, and [6-F]-Trp into proteins such as the *Escherichia coli* cyclic AMP receptor binding protein[6,7] and *Lactobacillus casei* dihydrofolate reductase[8] and used ^{19}F NMR to monitor which residues were influenced by ligand binding. Other workers have similarly examined fluoroaromatic amino acid containing samples of, e.g., galactose binding protein,[9] hemoglobin,[10] and lysozyme.[11] While these ^{19}F studies provided interesting information about conformational changes which accompany ligand binding, the structural information available was rather qualitative. In many of these studies large contributions to the ^{19}F shielding were attributed to electric field effects associated with van der Waal's dispersion interactions between the fluorine and neighboring atoms (separated by distance r_i).[5−16] This interaction depends directly on Σr_i^{-6} and was regarded as an approximate measure of the degree to which a fluorine containing amino acid was buried in the protein. However, more recent quantum mechanical calculations of ^{19}F shifts and shift tensors in aromatic molecules[17] as well as isotropic chemical shifts in fluorine-containing amino acids in proteins[18] have indicated that the observed chemical shifts can be evaluated *ab initio* without invoking a van der Waal's dispersion contribution to the shielding.

To date, the calculations of ^{19}F shielding constants in amino acids have all been concerned with aromatic amino acids, especially F-Trp. This is not surprising, since the available experimental NMR database on ^{19}F-labeled proteins is rich with such examples,[5,15] and in addition, F-Trp residues would seem

* Address correspondence to this author. Tel: 0181 959 3666. Fax: 0181 906 4477.
† National Institute for Medical Research.
‡ University of Sussex.
§ The Wellcome Research Laboratories.
⊥ University of Illinois at Urbana-Champaign.

|| Abbreviations: 2D, two-dimensional; DAST, diethylaminosulfur trifluoride; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DHFR, dihydrofolate reductase (EC 1.5.1.3); DMAP, 4-dimethylaminopyridine; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulphonate; [5-F]-Leu, (2*S*,4*S*)-5-fluoroleucine; HMQC, heteronuclear multiple quantum coherence; INEPT, insensitive nuclei enhanced by polarization transfer; TOCSY, two-dimensional total correlation spectroscopy.

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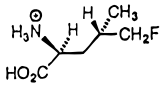
to be less likely to be involved in internal motion than would smaller aromatic amino acids, thus facilitating chemical shift calculations. The range of ^{19}F chemical shifts observed for the various fluorine-containing aromatic residues in proteins is very large, about 17 ppm.[11] This large chemical shift dispersion is useful in that it helps in resolving the relatively broad (~0.1−0.2 ppm) ^{19}F signals typically seen for larger proteins. However, no comparable studies have been carried out on fluorine-containing aliphatic amino acids. Presumably, this is because of the relative difficulty of obtaining suitable precursor molecules, which require nontrivial syntheses involving stereospecific incorporation of the ^{19}F-label. This can be illustrated by reference to a residue such as leucine. To simplify studies on an ^{19}F-containing leucine, it is preferable to have a leucine molecule containing only a single fluorine. Such a compound should be easier to incorporate into a protein than a multiply-labeled leucine and, after incorporation, should lead to minimal perturbation of the structure of the protein. It is also important for the fluorine atom to be present in a stereochemically well-defined position in the molecule, so that there is only one stereoisomer to consider. A fluorine-containing leucine which satisfies these criteria is the stereoisomer (2*S*,4*S*)-5-fluoroleucine (**1**), which can be prepared[19] using modifications of the procedures used previously for preparing (2*S*,4*R*)[5,5,5-$^2H_3$]-leucine.[23,24]



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To assess the potential utility of ^{19}F NMR studies of fluorine-containing aliphatic residues in proteins, we have prepared and examined ^{19}F NMR spectra of a sample of *L.casei* dihydrofolate reductase containing biosynthetically incorporated (2*S*,4*S*)-5-fluoroleucine ([5-F]-Leu DHFR). Such a study provides an indication of the range of ^{19}F chemical shifts which might be expected for aliphatic amino acids in proteins, and the results obtained permit an initial assessment of the possible factors controlling the ^{19}F shielding in aliphatic amino acids in proteins to be made.[18,20−22]

Materials and Methods

Synthesis of Intermediates. Scheme 1 indicates the various intermediates required for the synthesis of (2*S*,4*S*)-5-fluoroleucine (**1**). The details of the synthesis of the intermediates and final product (**1**−**10**) are given as supporting information.

Preparation of (2*S*,4*S*)-5-Fluoroleucine Dihydrofolate Reductase ([5-F]-Leu DHFR). *L. casei* DHFR containing [5-F]-leucine was prepared using an *E. coli* strain into which the gene for the *L. casei* enzyme had been cloned (*E. coli* NF1/pMT 702).[25] The cells were grown on a minimal medium containing 10 g/L D-glucose, 2 g/L ammonium sulfate, 14 g/L dipotassium hydrogen orthophosphate, 6 g/L potassium dihydrogen orthophosphate, 1 g/L sodium citrate, 0.2g/L magnesium sulphate, 50 mg/L ampicillin, 60 mg/L [5-F]leucine and 40 mg/L L-leucine. The *E. coli* NF1/pMT 702 strain also requires L-tryptophan (40 mg/L) and D-biotin (1 mg/L), and these were included in the medium.

Cells were grown at 30 °C in 8 L of minimal medium (in 1 L batches in 5 L flasks) until early log phase had been reached (A600 approximately 0.2). At this point, the incubation temperature was raised to 40 °C in order to induce DHFR expression. After approximately 20 h incubation at 40 °C, the cells were harvested (after this time the levels of DHFR start to decrease). The [5-F]-Leu DHFR was purified and assayed using the protocol described previously, with minor modification.[26]

The 1:1 binary complex of [5-F]-Leu DHFR with methotrexate (Sigma Chemical Company, St. Louis, MO) was prepared as described

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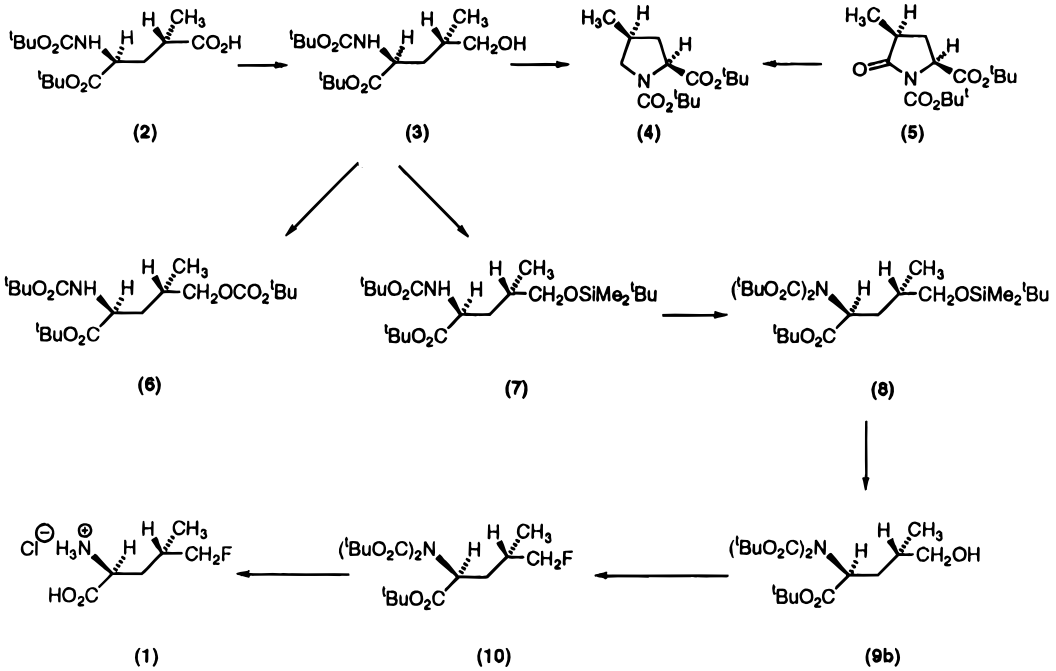
Scheme 1



previously.[27,28] The final NMR sample contained 1.7 mM DHFR-methotrexate dissolved in 0.6 mL H_2O/D_2O(90/10; v/v), 50 mM potassium phosphate, and 500 mM KCl, at pH 6.5.

NMR Spectroscopy. ^{1}H and ^{19}F NMR spectra of the [5-F]-Leu DHFR were recorded at 27 °C using a Bruker WM400 spectrometer operating at 400 and 377.6 MHz, respectively. ^{19}F chemical shifts were referenced with respect to an external sample of trifluoroacetic acid (see Figure 1), while the ^{1}H chemical shifts were referenced with respect to DSS (2,2-dimethylsilapentane-5-sulfonate). Chemical shifts are reported using the convention that high frequency, low field, paramagnetic, or deshielded values are more positive (International Union of Pure and Applied Chemistry, δ-scale). The ^{19}F-{^{1}H} decoupling experiment had the ^{1}H decoupler gated off during a 1s relaxation delay to allow recovery of the negative NOE effects. The pulse sequences used for the HMQC and 2D HMQC.TOCSY experiments were those described in the literature.[29,30]

For the synthetic work, (see supporting information) the ^{1}H-NMR spectra were recorded on Bruker WM 360 (360 MHz) and AMX 500 (500 MHz) spectrometers; ^{19}F NMR spectra on a Bruker WP 80 (75.38 MHz) spectrometer; and ^{13}C-NMR spectra on a Bruker AMX 500 (125.8 MHz) spectrometer. INEPT experiments were used to help assign ^{13}C-NMR resonances where necessary. *J* coupling values are given in Hz. Unless otherwise stated, residual solvent peaks were used as an internal reference in the ^{1}H nmr spectra.

Electrospray Mass Spectrometry. Five microliters of a desalted solution of [5-F]-Leu DHFR containing approximately 50−55 pmol μL^{-1} in 50% aqueous formic acid were infused into a VG Bio-BQ mass spectrometer (VG Organic, Altringham, U.K.) in an acetonitrile/water (50:50 v/v) matrix at a flow rate of 5 μL min^{-1}. In some runs, horse heart myoglobin was added as an internal mass calibrant. Ionization conditions were as follows: capillary voltage, 3.6 kV; HV lens voltage, 200 V; cone voltage, 21 V and source temperature, 80 °C. Spectra were recorded at a scan rate of 10 s/scan over a mass range of 550−1850 Da calibrated against a solution containing 20 pmol μL^{-1} of horse heart myoglobin. Transformation of the multiply charged electrospray spectra of the [5-F]-Leu DHFR samples to the zero charge state was performed using the algorithm supplied with the VG MassLynx data analysis software.

Other Analytical Methods Used in the Synthetic Work (See Supporting Information). Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotation measurements (given in units of 10^{-1} deg cm^2 g^{-1}) were obtained on a Perkin Elmer PE241 polarimeter, using a 1 dm path length microcell. IR spectra were recorded on a Perkin Elmer 1720 Fourier transform instrument. Mass spectra were recorded on Kratos MS80RF, MS50 and MS25 spectrometers, and accurate mass measurements were recorded on Kratos MS80RF and V67070 spectrometers. Microanalyses were performed by Mrs. P. Firmin (Wellcome Research Laboratories). Column chromatography was performed using Merck Kieselgel 60 (230−400 mesh ART 9385).

Results and Discussion

Synthesis of (2*S*,4*S*)-5-Fluoroleucine. The amino acid (2*S*,4*S*)-5-fluoroleucine (**1**) was prepared as previously reported in preliminary form[19] by the synthetic route outlined in Scheme 1 using the intermediate acid (**2**) from our synthesis of (2*S*,4*R*)-[5,5,5-$^2H_3$]leucine.[23,24] This compound appeared to be an excellent starting point for the synthesis, having the two chiral centres required of the target molecule. Reduction to the alcohol (**3**), fluorination using DAST, and deprotection might be expected to provide the desired target in a straightforward way.

In the event, the alcohol (**3**) was readily prepared as a colorless oil in 84% yield from the acid (**2**) by conversion to the mixed anhydride with isobutyl chloroformate and reduction with $NaBH_4$. However, reaction of the alcohol (**3**) with DAST at −40 °C gave a colorless oil in 63% yield with spectral characteristics which suggested that it was the protected *cis*-4-methylproline derivative (**4**). An authentic sample of this compound was therefore prepared independently in 65% yield by reduction of the pyroglutamate derivative (**5**) with borane dimethylsulfide in tetrahydrofuran. The product of the DAST reaction was spectroscopically identical to this authentic sample. It was evident that the urethane nitrogen in the intermediate formed from the alcohol (**3**) and DAST was sufficiently nucleophilic to compete with the fluoride ion, so that intramolecular cyclization yielded the proline derivative (**4**).

Since the cyclization reaction might be prevented if a second protecting group were present on nitrogen, we investigated the possibility of preparing a bis-urethane, in the first instance without protecting the primary alcohol group. Since no reaction was observed under the mild conditions recommended for exhaustive urethanylation,[31] we reacted the alcohol (**3**) with di-*tert*-butyl dicarbonate and DMAP in dioxan at 100 °C overnight. The product was obtained as a colorless oil in 58% yield and evidently contained a second *tert*-butoxycarbonyl group. The presence of an N*H* proton in the ^{1}H-NMR spectrum and the shift to lower field of the CH_2OR protons, however, suggested that O-acylation had occurred to give the product (**6**). This was confirmed by the other spectral data. The alcohol function was therefore protected by reaction with *tert*-butyldimethylsilyl chloride, triethylamine, and DBU in dichloromethane. The silyl ether (**7**) was obtained as a colorless oil in quantitative yield. In order to achieve perurethanylation of the product (**7**), it was necessary to heat it to 100 °C in dioxan with triethylamine and DMAP whilst adding excess di-*tert*-butyl dicarbonate dropwise. The fully protected product (**8**) was then obtained as a colorless oil in 95% yield.

When deprotection of the silyl ether was attempted using tetrabutylammonium fluoride in tetrahydrofuran at room temperature, the product alcohol (**9a**), obtained in 51% yield, was shown by its ^{1}H and ^{13}C NMR spectra to be a 1:1 mixture of diastereoisomers. This was ascribed to the fact that, when the oxyanion is produced by fluoride ion deprotection, it can act as a base for the intramolecular removal of the C-2 hydrogen thus leading to epimerization at C-2. In the hope of quenching the intermediate anion before it caused epimerization, the silyl ether (**8**) was stirred at room temperature with tetrabutylammonium fluoride in tetrahydrofuran in the presence of acetic acid. The product was obtained from this reaction as a white solid in 94% yield. This was evidently a single diastereoisomer of the *N*,*N*-di-*tert*-butoxycarbonyl alcohol (**9b**), as shown by both ^{1}H- and ^{13}C-NMR spectra.

We were now in a position to attempt to replace the alcohol group with fluorine, without intramolecular cyclization, by using the N-diprotected substrate. Although reaction with DAST at −40 °C for 1 h gave mixtures, a 65% yield of the desired product (**10**) was obtained by leaving the alcohol (**9b**) with excess DAST in the presence of triethylamine for several days at room temperature. This compound was a colorless oil, which could be deprotected to yield (2*S*,4*S*)-5-fluoroleucine hydrochloride (**1**) in 86% yield, by stirring in 6 N HCl for 4 days at room temperature.

Incorporation of [5-F]-leucine into DHFR. *L. casei* DHFR containing (2*S*,4*S*)-5-fluoroleucine was prepared using an *E.coli* strain into which the gene for the *L.casei* enzyme had been cloned (E.coli NF1/pMT702).[25] Preliminary experiments showed that inclusion of 100 mg/L of 5-fluoroleucine in the growth medium was almost completely inhibitory to growth but further experiments using mixtures of 5-fluoroleucine and leucine showed that a mixture containing 60 mg/L [5-F]leucine and 40 mg/L leucine gave good growth (only 25% growth inhibition),

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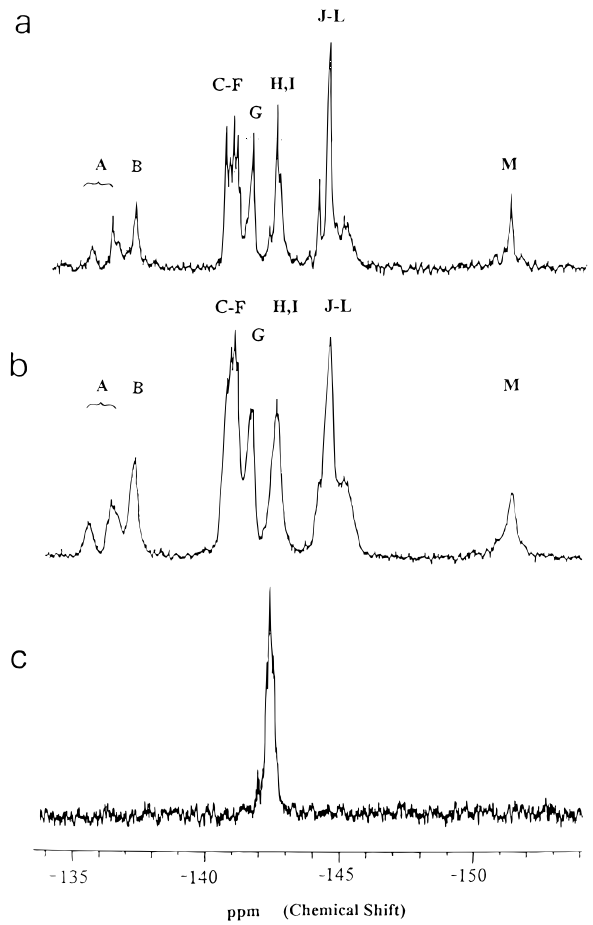

Figure 1. 377.6 MHz ^{19}F NMR spectra of [5-F]-Leu DHFR complexed with one equivalent of methotrexate at 27 °C (a) ^{1}H decoupled spectrum (43 300 scans); decoupler gated-off during the 1.0 s relaxation delay; (b) ^{1}H-coupled spectrum (78 000 scans). Relative intensities (assuming the total intensity corresponds to 13 fluorines) A, 1.0; B, 1.0; C−G, 4.7; H−I, 1.8; J−L, 3.6; M, 0.9; (c) ^{1}H decoupled spectrum of sample denatured with 6 M guanidine hydrochloride (169 000 scans). Spectral width, 50 000 Hz; acquisition time, 0.328 s; relaxation delays, (a) 1.0 s (b) 1.7 s (c) 1.0 s; pulse widths, (a) 32° (b) 45° (c) 45°; 32K data points.

and this mixture was used in the final growth medium. The purified [5-F]-Leu DHFR had 60 ± 5% of the activity of the native enzyme.

Electrospray Mass Spectrometry Studies. The results from the electrospray mass spectrometry (Figure 2) indicated that the DHFR sample contains species with up to six [5-F]-Leu residues incorporated into a single protein, with the maximum in the population having two [5-F]-Leu residues (see signal for 18345.0 Da in Figure 2). This distribution of species is that statistically expected for a sample containing 22% incorporation of [5-F]-Leu (even though the growth medium contained 63% of [5-F]-Leu). The signals in the 18440 to 18530 Da region of the transformed mass spectrum arise from the presence of 20−30% of the *N*-methionine terminus form of the protein.

NMR Studies on [5-F]-Leu DHFR. In previous NMR studies on the complex of *L. casei* DHFR with methotrexate, we reported ^{1}H and ^{13}C resonance assignments, including stereospecific assignments, for the 13 leucine residues.[28,33,34] In those studies, each amino acid residue gave rise to a single set of resonances, indicating that the complex exists (on average) as a single conformational state.

Figure 1a shows the proton decoupled ^{19}F NMR spectrum of the [5-F]-Leu DHFR in its complex with methotrexate. There are 12 fairly sharp signals (A−M), one of which corresponds to two overlapped signals (K,L) having similar chemical shifts. The ^{19}F chemical shifts are seen to extend over a very large range, 15 ppm, almost as large as that found with aromatic fluorine-containing amino acids in proteins.[11] There are also a few broad signals underlying the narrow signals present in the spectrum. These small bands appear to be composed of several overlapping signals with different chemical shifts. Figure 1b shows the ^{19}F spectrum of the complex recorded without ^{1}H decoupling, and intensities measured in this spectrum indicate that some of the signals have nonintegral intensities. These nonintegral intensities are not due to relaxation effects because spectra recorded with different relaxation delays (1 and 1.7 s) showed no noticeable differences in intensities (the relaxation times of the different fluorines are in the range 0.20 to 0.35 s as estimated by inversion recovery experiments).

There are several possible causes of the nonintegral intensities, relating to problems associated with sample heterogeneity. The mass spectrometry results indicate that there is a mixture of DHFRs containing up to six [5-F]-Leu residues. If, as expected, there is a random distribution of the [5-F]-Leu residues incorporated, then each different combination would result in each [5-F]-Leu at a specific position being formally nonequivalent, and this could clearly lead to ^{19}F chemical shift differences. Furthermore, if some of the [5-F]-Leu substituted DHFR molecules are unstable or unable to fold to the correct native structure (required for the methotrexate affinity column purification to work), this could also lead to a nonstatistical [5-F]-Leu distribution. Another possible source of heterogeneity could arise if the three different leucine transfer RNAs are differentially charged with [5-F]-Leu (three different codons[20] are used for leucine residues in *L.casei* DHFR). An additional source of heterogeneity comes from the presence of 20−30% of the *N*-methionine terminus form of the protein, as detected in the transformed electrospray mass spectrum. This, for example, could account for the signals with nonintegral intensities in the −135 to −137 ppm region of the ^{19}F NMR spectrum. The intensities of the resolved and overlapped signals given in the Figure 1b caption are within ±20% of the values expected for the 13 [5-F]-Leu residues contributing to the different signals, which allows us to make an initial analysis of the results.

Figure 1c shows the ^{19}F spectrum of [5-F]-Leu DHFR after denaturation with 6 M guanidine hydrochloride. A broad signal (width 0.6 ppm) centered at the chemical shift of a protected [5-F]-Leu (compound **10** used as peptide model) indicates that the fluorine has not been scrambled into other residues. The observed signal is as expected for several [5-F]-leucines showing small shielding differences reflecting their different immediate neighbors in the sequence of the unfolded protein.

A ^{1}H spectrum of the [5-F]-Leu DHFR complex was also recorded (not shown) and the overall features were very similar to that of the unlabeled DHFR complex, indicating that the protein is retaining its proper folded structure. This is not surprising in view of the high activity measured for the protein. All the high field methyl signals were detected at their characteristic chemical shifts, but the Leu signals had modified intensities. Intensity measurements of the relevant methyl signals in Leu-113 and Leu-118 indicated that 20−25% of the leucines had been substituted by [5-F]-leucine in agreement with the electrospray mass spectrometry results.

The ^{19}F/^{1}H HMQC spectrum of the sample is shown in Figure 3. Two of the protons in [5-F]-leucine have a large H−F spin−spin coupling with the 5-F nucleus, namely, the two nonequivalent protons in the CH_2F group ($^{2}J_{HF} \sim 50$ Hz[32]). A large H−F spin−spin coupling can also occur with the γ-CH proton ($^{3}J_{HF} = 0$ to 40 Hz), depending on dihedral angle.[32] Thus, at each

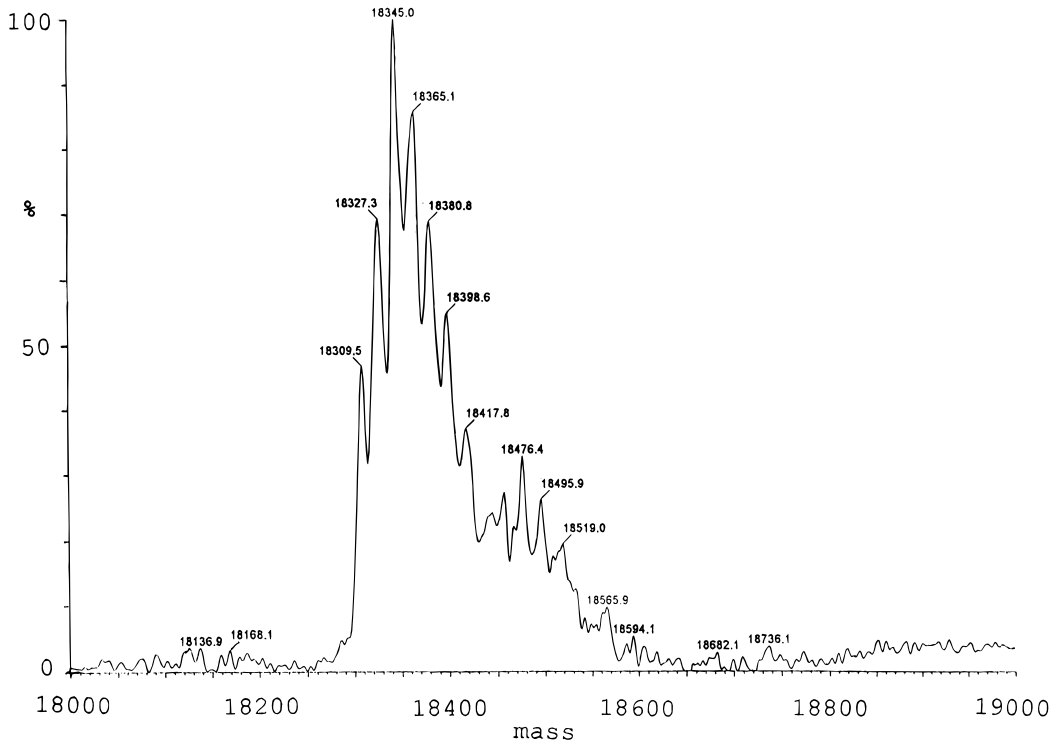

Figure 2. The electrospray mass spectrum of the [5-F]-Leu DHFR sample transformed to the true mass scale. The wild-type enzyme has a calculated mass of 18307.6 Da with an observed mass at 18309.5 Da. The spectrum shows a series of mass shifts with an average of 18 Da corresponding to a maximum of six [5-F]-Leu residues per DHFR molecule. A second series with a maximum of 18476.4 Da, corresponding to the presence of an *N*-methionine terminus, is also present.

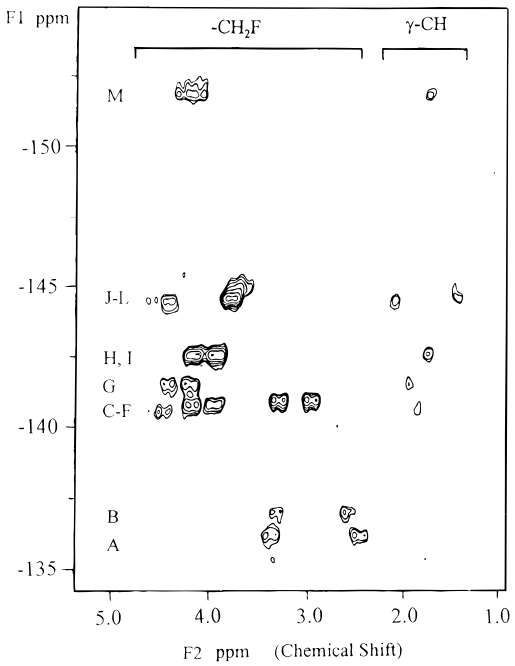

Figure 3. 9.4 Tesla ^{19}F-^{1}H HMQC spectrum of [5-F]-Leu DHFR complexed with 1 equiv of methotrexate at 27 °C. The F_1 (^{1}H) dimension had a spectral width of 4000 Hz recorded with an acquisition time of 0.256 s, and the F_2 (^{19}F) dimension had a spectral width of 10 417 Hz, recorded with 256 increments and 672 scans/increment.

^{19}F frequency in the HMQC spectrum, one might expect to see correlations to these three protons. Examination of the HMQC spectrum shown in Figure 3 reveals that such correlations are indeed observed for most of the ^{19}F resonances (peaks C to M), while two of them (A and B) only show correlations with the two geminal protons. Because the size of the three bond coupling constant to the γ-H depends on the corresponding dihedral angle, or more precisely its time-averaged value, the three bond couplings can sometimes be very small or even zero, in which case the vicinal H−F correlations would go undetected. A comparison of the HMQC spectrum with a 2D HMQC-TOCSY spectrum (not shown) indicates that there are five additional signals in the latter, corresponding to δ-methyl groups in five of the 13 leucine residues (at 0.50, 0.55, 0.68, 0.87, and 1.10 ppm). These correlations depend to some extent on the size of the $H_\gamma-H_\delta$ coupling constants, and those that involve the CH_2F H_δ protons will be sensitive to the corresponding dihedral angle, χ_3.

A complete set of stereospecific ^{1}H resonance assignments for the leucine residues in the DHFR-methotrexate complex has been obtained previously.[33,34] In this work, we have made approximate estimates of the corresponding ^{1}H shifts for the complex formed with the [5-F]-Leu DHFR by considering the chemical shifts in the unlabeled complex, in conjunction with the incremental shift changes observed for protons in free leucine when a fluorine atom is introduced into the δ-methyl group (the substituent effect on fluorination is +4.3 ppm for a CH_2F group[19]). The observed range of ^{1}H chemical shifts for the CH_2F protons fall within the range predicted by the above simple method (observed shifts in the range of δ = 2.5−4.5 ppm; estimated shifts δ = 2.4 to 4.5 ppm). For the complex with native DHFR, the methyl protons of two leucines (Leu-113 and Leu-118) experience large ring-current shieldings and the estimated shifts for [5-F]-Leu DHFR suggest that two groups of CH_2F protons experience similar ring-current shifts. Based on these considerations signals A and B are tentatively assigned to Leu-113 and Leu-118 but not on a one-to-one basis.

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Table 1. Calculated Effects of HF Molecules on R−^{19}F Shielding (R = nPr, nBu, Ph) as Models for Electrostatic Field Effects in Proteins

	$\Delta\delta^b$ (ppm)		
RF···HF distance[a] (Å)	R = nPr	R = nBu	R = Ph
3.0	2.886 (4.752)	2.754 (4.316)	5.984 (4.035)
3.2	2.419 (3.445)	2.319 (3.150)	nd
3.4	2.035 (2.357)	1.956 (2.171)	nd
3.6	1.726 (1.516)	1.672 (1.408)	nd
4.0	1.288 (0.518)	1.250 (0.490)	nd
5.0	0.714 (0.012)	0.750 (−0.037)	1.704 (0.011)

[a] Separation between ^{19}F atom in RF and H atom in HF. [b] Shielding increase on HF interaction. The counterpoise corrections used to minimize basis set superposition errors are shown in parentheses.

^{19}F Chemical Shifts. We now consider some of the factors which can contribute to the chemical shift range observed in the [5-F]-Leu DHFR spectra. The overall range of chemical shifts is 15.2 ppm. This is a very large value, being almost as large as the largest range observed for aromatic amino acids in any protein, the 16.8 ppm seen in [4-F]-Trp ^{19}F-hen egg white lysozyme/$(NAG)_3$.[11] Previously, it has been suggested that electric field effects are a major contributor to ^{19}F shielding in proteins,[20] basically because aromatic C−F bonds are highly polarizable, and there are large electrostatic fields in the interior of a protein. The ^{19}F chemical shift in an aromatic system such as fluorobenzene or fluorophenylalanine responds to an electric field, and the change in chemical shift with field is about 2000 ppm per atomic unit of field (an atomic unit of field is 5×10^9 V/cm). Since electric fields in proteins can vary by 0.008 au,[18,20] a maximum shift range of 2000×0.008, i.e., 16 ppm is expected from this uniform field component. In the *E. coli* galactose binding protein, good accord between experiment and calculation was obtained using this general approach (which included molecular motion and the nonuniform field contributions to shielding)[18] as well as the gauge including atomic orbitals (GIAO) method.[36] For $-CH_2F$ groups, however, the change in chemical shift is expected to be much less than with aromatic fluorines, because the C−F bond is less polarizable in the saturated system. The overall shift range will thus be scaled down by approximately the ratio of the shielding derivatives with respect to a uniform field—the shielding polarizabilities or A-terms.[20] Based on previous work with CH_3F and fluorobenzene, the maximum total range expected will be $16.8 \times 551.4/1884.5$, or about 5 ppm. This assumes that the shielding response to an electric field is very similar in CH_3F and F-leucine, which we believe will be the case. It also assumes that the $-CH_2F$ group has about the same mobility as a F-Trp, that is it is essentially static.

To further investigate the effects of electrostatic fields on -CH_2F shielding, we have carried out *ab initio* calculations of ^{19}F shielding in fluoropropane and fluorobutane molecules perturbed by an adjacent H−F molecule, to simulate the effect of electric fields on F-Leu shielding. The results of these calculations, performed using the Texas-90/93 suite of programs,[37] are shown in Table 1, together with corresponding earlier results for fluorobenzene, again electrically perturbed by HF. The results for fluoropropane and fluorobutane are very similar, but the field-induced shifts are clearly much smaller than those seen in the case of fluorobenzene. These *ab initio* results again indicate that electric field effects are unlikely to explain the 15 ppm range of chemical shifts we observe in [5-F]-Leu DHFR, Figure 1a. The electric field induced shifts for the aliphatic residues interacting with HF are only 45% of those seen with the aromatic species, implying a maximal 7−8 ppm contribution to the observed chemical shift range. In addition, in the $-CH_2F$ group, it is likely that there will often be rotation about Cδ-Fϵ. In this case, there will be extensive motional averaging of the electrical interaction, as the C−F vector reorients in the electric field of the protein. On average, this will reduce the overall electrical contribution to shielding.

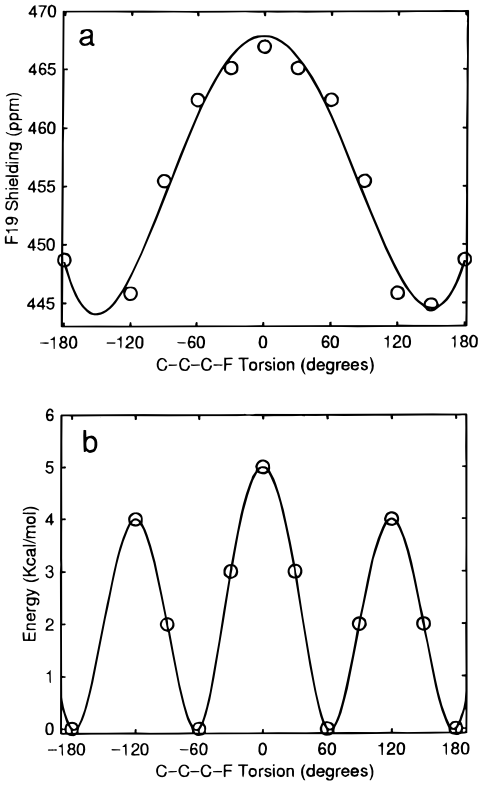

Figure 4. Effects on ^{19}F shielding and energy in 1-fluoropropane as a function of the C−C−C−F torsion angle: (a) ^{19}F chemical shifts and (b) torsional potential. A 6-311G++(2d,2p) basis set was used on F, 6-311G elsewhere.

We therefore need to seek alternative effects which may cause substantial contributions to shielding since the results of earlier calculations of ^{19}F shifts in fluoroaromatic amino acids in proteins suggested that van der Waal's dispersion contributions to the shielding were unlikely to be important.[17,18] For aliphatics, one potentially important contribution which is unavailable in a F-Trp or F-Phe residue is from electronic structural effects, due to local conformational differences in the leucine side chains. For example, in ^{13}C NMR, the effects of ϕ,φ on ^{13}Cα dominate its shielding,[38] and in small molecules, the so-called γ-gauche effect[39] has been known for many years, and contributes up to a ∼4−5 ppm shielding for gauche over trans conformers in ^{13}C NMR. We have therefore investigated whether such conformational differences might be significant contributors to the ^{19}F shift nonequivalencies we observe in [5-F]-Leu DHFR.

We show in Figure 4a ^{19}F shielding in l-fluoropropane as a function of the C−C−C−F dihedral angle, χ. While this

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molecule is considerably smaller than [5-F]-Leu, it nevertheless carries the same γ-methyl interaction, and should serve as a good model for conformational effects on shielding. At the fully eclipsed configuration ($\chi = 0°$), we observe maximum shielding, as in ^{13}C NMR. Such fully eclipsed conformers are expected to be very rare, however, since they have a high energy, as shown in the torsional potential graph, Figure 4b. The low energy conformers, as expected, are the $\chi = \pm 60°$ and 180° forms, also shown in Figure 4b. Interestingly, the chemical shift difference between these two stable conformers is about 10 ppm, Figure 4a. This is clearly a very large value, and these results suggest that side-chain conformational effects may make a major contribution to ^{19}F shielding in [5-F]-Leu in DHFR, and proteins in general.

Similar calculations on side-chains of [5-F]-leucine amide also indicate substantial ^{19}F shielding differences between rotameric states (see supporting information). In the most populated [5-F]-leucine side-chain conformer ($\chi_1 = -60°$, $\chi_2 = 180°$), the shielding of the χ_3 "60°" (gauche) conformer is estimated to be 12 ppm above that seen in the "180°" (trans) conformer, due to the γ-gauche shielding effect of the γ-methyl group. If the CH_2F group occupies the χ_3 rotameric states unequally then there could be up to a 12 ppm contribution to the observed chemical shift non-equivalences seen experimentally. Related effects are seen with the second most populated side-chain conformation ($\chi_1 = 180°$, $\chi_2 = 60°$) as well as with the (2*S*,4*R*)[5-F]-leucine amide (data not shown).

Our experimental and theoretical results are thus consistent with the idea that there can be a considerable contribution to ^{19}F shielding due to the γ-gauche effect if one or more CH_2F groups exist as a mixture of conformers with a large population of the gauche conformation. The effects of F-substitution (and possible hydrogen bonding) may increase barriers in some cases, favouring a particular χ_3 conformation. Such effects are not unprecedented, since, e.g., in the case of [2-F]-phenylalanine-labeled hen egg white lysozyme (HEWL), all three F-Phe residues were locked into two different conformations,[10] with six peaks being observed for the three labeled fluorines. Such peak doubling was not observed for the [3-F]-Phe HEWL, clearly indicating that F-substitution can indeed influence local structure.[11]

Whether this γ-effect is the major contributor to the observed ^{19}F shielding range will require further work in which complete side-chain conformations are determined experimentally. What our initial calculations do show, however, is that unlike the situation with fluoroaromatic amino acids in proteins, electric field effects are unlikely to dominate aliphatic ^{19}F-amino acid chemical shift nonequivalences in proteins due to folding. Fluoroaromatic amino acids have highly polarizable fluorines (due to orbital overlaps with the π-electron cloud), and conformational transitions in general will not change the local electronic structure significantly. Fluorinated aliphatic amino acids, on the other hand, have much less polarizable fluorines, but can undergo conformational changes which do affect the electronic structure, in particular via the γ-gauche effect. A 5−8 ppm maximal shift range due to electrostatics, up to 12 ppm due to γ-gauche conformational effects, plus up to ∼2 ppm due to ring current effects can thus contribute to the $-CH_2{}^{19}F$ chemical shifts in proteins. Further progress in understanding the large chemical shift range seen for this fluoro-aliphatic amino-acid in DHFR will necessitate the assignment of each [5-F]-Leu resonance, and a determination of each side-chain conformation.

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Supporting Information Available: Experimental details, chemical shift calculations, and figure representing effects of torsion angle on ^{19}F shielding and relative energy (13 pages). See any current masthead page for ordering and Internet instructions.

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