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A Mutagenesis-Free Approach to Assignment of ^{19}F NMR Resonances in Biosynthetically Labeled Proteins

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Fluorine (^{19}F) NMR is ideally suited to the study of protein folding or unfolding, ligand binding, enzymatic action, and internal motions.[1,2] In comparison with traditional labels such as ^{1}H, ^{13}C, and ^{15}N, ^{19}F spin probes give rise to a wide range of chemical shifts associated with the unique local electronic environments in folded proteins.[3−5] Moreover, ^{19}F can be readily incorporated by biosynthetic means, often using nonauxotrophic bacterial strains with an efficiency which depends on both the protein and amino acid.[6] Perturbations to protein structure and function are minimal[7,8] or can be made so by employing a fractional labeling strategy.[9] The most common monofluorinated probes include tyrosine, phenylalanine, and tryptophan, where an induced-auxotrophy approach produces uniform labeling with efficiencies of ~95%.[10,11]

Assignment of fluorine resonances is often not straightforward and can only be achieved by resorting to site-directed mutagenesis. Generally, a series of mutants and corresponding spectra are obtained wherein each labeled residue is separately replaced with a structurally similar amino acid; for example each occurrence of fluorotyrosine might be substituted for phenylalanine.[2,12] Alternatively, where such substitutions result in gross changes in either the protein structure or the resulting ^{19}F NMR spectrum, a residue within van der Waals contact of a particular fluorine nucleus may be mutated to affect a change in the fluorine chemical shift.[2] However, this so-called nudge mutation method requires *a priori* knowledge of the protein structure. Mutagenesis approaches rapidly become time-consuming and problematic due to spectral overlap for proteins possessing many labeled residues. Here, we propose an approach which reduces spectral overlap and avoids the use of mutagenesis in assigning ^{19}F resonances of a fluorotyrosine enriched protein. The approach requires that the protein is first biosynthetically labeled with a ^{13}C and ^{15}N-enriched version of 3-fluorotyrosine. Through a combination of INEPT and COSY based transfers which utilize the large ^{19}F−^{13}C and ^{13}C−^{13}C scalar couplings, it is possible to correlate the ^{19}F resonances with the ^{15}N, ^{1}H, and ^{13}C resonances of the backbone, thereby accomplishing the assignments. Figure 1 outlines the CT-HCCF-COSY pulse sequence which achieves a transfer from the delta proton of the aromatic ring to the fluorine spin at the 3-position and is similar to earlier ^{13}C−^{1}H experiments.[13] The assignment is then completed using standard NMR pulse sequences[14] which correlate the side chain resonances to the backbone ^{15}N, ^{1}H, and ^{13}C chemical shifts (*vide infra*).

Synthesis of ^{13}C,^{15}N-3-fluoro-L-tyrosine was achieved in one step by electrophilic fluorination of ^{13}C,^{15}N-L-tyrosine. Selectfluor is a recently developed safe alternative to traditional electrophilic fluorine sources such as F_2 and XeF_2.[15] The reduced activity of this reagent permits monofluorination under mild conditions, alleviating the problem of producing a distribution of mono-, di-, and trifluorinated products, which would otherwise reduce the yield of the desired monofluorinated variant. Selectfluor was added to the amino acid dissolved in 80/20 acetonitrile/water in a 3:1 stoichiometric ratio and reacted for 2.5 h at 80 °C. After removal of solvent *in vacuo*, the product was redissolved in water adjusted to pH 2 with formic acid and separated from reagent byproducts using a gravity flow Sepabead SP850 column where a mixture of fluorinated and nonfluorinated tyrosine was eluted using 15% acetonitrile in water. RP-HPLC on a C-18 column achieved separation of fluorinated and nonfluorinated amino acids. Lyophilization then yielded pure ^{13}C,^{15}N-3-fluoro-L-tyrosine in 79% isolated yield. A cost-effective feature of this reaction and purification scheme is the ability to recycle nonfluorinated ^{13}C,^{15}N-L-tyrosine for subsequent fluorination. Production of 33 mg/L purified protein required 18 mg/L of ^{13}C,^{15}N-3-fluoro-L-tyrosine.

Figure 1. CT-HCCF-COSY pulse scheme. Narrow (wide) pulses are applied with 90°(180°) flip angles and phases, *x*, unless otherwise indicated. $\tau_H = 1.6$ ms, $\tau_F = 0.9$ ms, $\Delta_{CH} = 1$ ms, $\Delta_{CF} = 0.7$ ms, and $T = 3.8$ ms, while a 2 kHz ^{13}C WALTZ-16 decoupling field is used. Phase cycle $\phi_1 = x, -x$; $\phi_2 = 4(x), 4(-x)$; $\phi_3 = 2(x), 2(-x)$; rec $= x, -x, x, 2(-x), x, -x, x$.

The assignment experiments are demonstrated on Ca^{2+}-bound ^{13}C,^{15}N 3-fluoro-L-tyrosine-enriched calmodulin (CaM) from *Xenopous laevis* using previously described expression[11] and purification protocols.[16] Although CaM has only two tyrosine residues, Y99 and Y138, located in the C-terminal lobe, its size and tumbling time (~9 ns) were deemed ideal to test the robustness of NMR pulse sequence schemes involving magnetization transfer along the aromatic side chain of medium sized proteins. The corresponding resonances are separated by 0.5 ppm. (^{15}N,^{1}H) HSQC spectra of fluorinated and nonfluorinated CaM confirmed the fluorinated protein was fully calcium loaded and retained the native structure. The assignment strategy, shown in Figure 2, begins with the correlation of each fluorine resonance to the corresponding delta proton via three one-bond transfers using the pulse scheme shown in Figure 1. The analogous CT-FCCH-COSY experiment differs only in the ordering of the frequency channels and the inclusion of a presaturation period for water. Sensitivity associated with the HCCF or FCCH experiment is proportional to $\gamma_1\gamma_2^{3/2}(\Delta\nu_1/\Delta\nu_2)$, where γ_1, γ_2, $\Delta\nu_1$, and $\Delta\nu_2$ represent the gyromagnetic ratios and line widths of the starting and detected nuclei, respectively. Therefore, if we consider the observed Hδ and ^{19}F line widths, we estimate that the sensitivity of the FCCH experiment is 1.9 times greater than that of the HCCF, although the latter sequence has the

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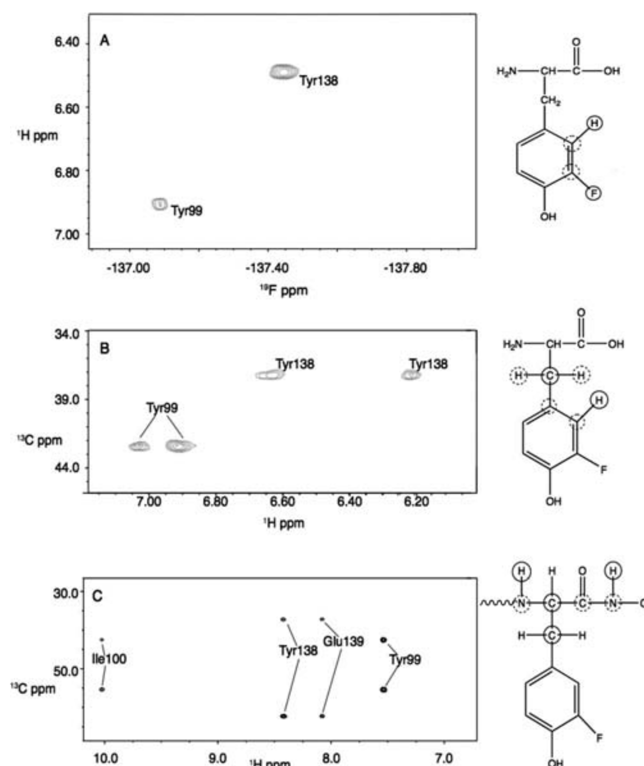

Figure 2. Spectra used to assign fluorine resonances of 1 mM CaM enriched with ^{13}C,^{15}N-3-fluoro-L-tyrosine. Evolved nuclei are indicated with full circles, while dashed circles are used for nuclei involved in magnetization transfer. (A) CT-HCCF-COSY (B) (Hβ)Cβ(CγCδ)Hδ (C) HNCACB. Experiments were run at 37 °C and recorded on a 600 MHz Varian Inova spectrometer equipped with a HFCN quad probe (Varian Inc., Palo Alto, CA).

advantage that there is no water signal. Connection to the backbone resonances is achieved, as shown in Figure 2B and 2C, using the (Hβ)Cβ(CγCδ)Hδ[17] and the HNCACB experiments.[14] The amide proton and nitrogen chemical shift data for calmodulin were obtained from previously published data (BMRB 6541) but can in principle can be obtained using the same sample if expressed under the conditions required to produce uniform ^{13}C and ^{15}N labeling. In some cases, published amide ^{15}N and ^{1}H chemical shifts may differ slightly from experimental values obtained on a fluorinated protein. In such situations the HNCACB and HNCO experiments help to verify assignments by utilizing the chemical shifts of the neighboring Cα, Cβ, and amide ^{15}N and ^{1}H nuclei.

The use of ^{13}C,^{15}N-enriched fluorotyrosine greatly enhances possibilities associated with ^{19}F NMR. First, (^{13}C,^{19}F) CT-HSQCs of CaM can be acquired in a matter of minutes (data not shown), reducing spectral overlap in biosynthetically labeled proteins. The ^{13}C−^{19}F spin pair can also be used to evaluate local order parameters and discern slow and fast motions through T_1, T_2, and ^{13}C−^{19}F NOE measurements. Finally, the ~50−75 ppm CSA common in fluoroaromatic amino acids[18] suggests that TROSY effects may be useful at low field strengths, resulting in improved line widths.

In summary we have demonstrated that mutagenesis-free ^{19}F NMR assignments of ^{13}C,^{15}N-enriched 3-fluoro-L-tyrosine can be routinely performed in protein studies. The ^{13}C−^{19}F pair is also expected to be useful via (^{13}C,^{19}F) HSQCs in reducing spectral overlap and in dynamics studies of side chains. The one-step synthesis procedure employed produces the fluorinated amino acid in high yield and allows for the recycling of nonfluorinated starting material for subsequent fluorination, making this scheme cost-effective compared to previously reported methods.[19,20] The CT-HCCF-COSY experiment produces high quality spectra, while additional 2- and 3-D NMR experiments may be used to correlate side chain resonances with those of the backbone. Similar approaches may be possible with other fluoroaromatics. For example, ^{13}C,^{15}N-enriched phenylalanine may be fluorinated after an intermediate nitration and reduction step.

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Supporting Information Available: Synthetic reaction scheme and NMR data for synthesis of ^{13}C,^{15}N 3-fluoro-L-tyrosine. This material is available free of charge via the Internet at http://pubs.acs.org.

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