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ARTICLE

Approaches to the assignment of ^{19}F resonances from 3-fluorophenylalanine labeled calmodulin using solution state NMR

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Abstract Traditional single site replacement mutations (in this case, phenylalanine to tyrosine) were compared with methods which exclusively employ ^{15}N and ^{19}F-edited two- and three-dimensional NMR experiments for purposes of assigning ^{19}F NMR resonances from calmodulin (CaM), biosynthetically labeled with 3-fluorophenylalanine (3-FPhe). The global substitution of 3-FPhe for native phenylalanine was tolerated in CaM as evidenced by a comparison of ^{1}H-^{15}N HSQC spectra and calcium binding assays in the presence and absence of 3-FPhe. The ^{19}F NMR spectrum reveals six resolved resonances, one of which integrates to three 3-FPhe species, making for a total of eight fluorophenylalanines. Single phenylalanine to tyrosine mutants of five phenylalanine positions resulted in ^{19}F NMR spectra with significant chemical shift perturbations of the remaining resonances, and provided only a single definitive assignment. Although ^{1}H-^{19}F heteronucleclear NOEs proved weak, ^{19}F-edited ^{1}H-^{1}H NOESY connectivities were relatively easy to establish by making use of the $^{3}J_{FH}$ coupling between the fluorine nucleus and the adjacent fluorophenylalanine δ proton. ^{19}F-edited NOESY connectivities between the δ protons and α and β nuclei in addition to ^{15}N-edited ^{1}H, ^{1}H NOESY crosspeaks proved sufficient to assign 4 of 8 ^{19}F resonances. Controlled cleavage of the protein into two fragments using trypsin, and a repetition of the above 2D and 3D techniques resulted in unambiguous assignments of all 8 ^{19}F NMR resonances. Our studies suggest that ^{19}F-edited NOESY NMR spectra are generally adequate for complete assignment without the need to resort to mutational analysis.

Keywords ^{19}F NMR · Calmodulin · 3-fluorophenylalanine · ^{1}H-^{19}F NOESY-HSQC

Abbreviations

1D	One-dimensional
2D	Two-dimensional
3D	Three-dimensional
CaM	Calmodulin
CSA	Chemical shift anisotropy
DNase	Deoxyribonuclease
RNase	Ribonuclease
IPTG	Isopropyl β-D-1-thiogalactopyranoside
EDTA	Ethylenediaminetetraacetic acid
NMR	Nuclear magnetic resonance

Introduction

^{19}F NMR is ideally suited to the study of changes in protein structure and dynamics related to folding and unfolding, enzymatic action, ligand binding and internal motions (Danielson and Falke 1996; Gerig 1994; Phillips et al. 1991). In comparison to traditional ^{1}H-^{13}C or ^{1}H-^{15}N two-dimensional NMR, ^{19}F NMR approaches benefit from lack of background signal, large chemical shift range, large scalar couplings for efficient magnetization transfer to ^{13}C

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or ^{1}H nuclei, and a high magnetogyric ratio (Gakh et al. 2000). Of particular importance in protein studies, ^{19}F NMR chemical shifts are sensitive to their local electrostatic environment and van der Waals packing (Chambers et al. 1994; Feeney et al. 1996). A variety of mono-, di- and per-fluorinated amino acids are commercially available, and are often cheaper than their ^{13}C or ^{15}N sidechain enriched counterparts. Furthermore, mono-fluorinated amino acids are introduced with high efficiency ($\sim$95%) via biosynthetic means, often using non-auxotrophic strains with little compromise to expression yields (Anderluh et al. 2005; Evanics et al. 2007). Structural and functional perturbations arising from the incorporation of mono-fluorinated amino acids are readily evaluated using a variety of techniques, most commonly NMR, circular dichroism spectroscopy, and fluorescence; and are generally found to be minor (Li and Frieden 2007; Xiao et al. 1998).

Despite the suitability of ^{19}F NMR to the study of protein structure and dynamics, assignments are generally established via site-directed mutagenesis techniques. In this study we evaluate the merits of site-directed mutagenesis strategies and demonstrate the use of ^{19}F and ^{15}N-edited 2D and 3D NMR experiments in the assignment of ^{19}F resonances associated with a protein uniformly labeled with 3-fluorophenylalanine.

The most common assignment strategy used in ^{19}F NMR studies involves direct replacement of the fluorinated residue with a structurally similar amino acid; for example, each occurrence of a fluorophenylalanine probe might be substituted by tyrosine using site-directed mutagenesis techniques. Although in most cases the resulting perturbations are minor, these effects are amplified in the corresponding ^{19}F NMR spectra due to the sensitive nature of the chemical shielding tensor. Frequently, the outcome of this replacement strategy precludes complete spectral assignment due to gross chemical shift perturbations of the remaining fluorine resonances (Feeney et al. 1996; Okano et al. 1998). Under such circumstances, the nudge mutation method is often employed which involves the mutation of a residue known to be within van der Waals contact of a particular fluorine probe to affect a change in it's chemical shift (Drake et al. 1993). This method, although often less perturbing, requires a priori knowledge of the protein structure. In general, both strategies require a complete series of mutants, which is costly and labor-intensive, while assignments using mutagenesis alone are often incomplete. Supplementary NMR experiments, which focus on local topology and ^{19}F relaxation properties, can be employed to resolve ambiguities. For example, in a recent study of the native and denatured states of green fluorescent protein, the authors complemented spectra from a complete series of single replacement mutants with ^{19}F T_1-relaxation measurements, as well as ^{19}F photo-CIDNP data to achieve complete assignment of ten 3-fluorotyrosine resonances (Khan et al. 2006).

Herein, we consider a mutation-free approach to the assignment of ^{19}F resonances from a 148 residue protein uniformly labeled with 3-fluorophenylalanine. Established triple resonance side chain assignment strategies often rely on uniform carbon enrichment to facilitate magnetization transfer between a given sidechain nucleus and those associated with the protein backbone. Such a method has previously been developed for the assignment of ^{19}F resonances in protein studies which employ 3-fluorotyrosine (Kitevski-LeBlanc et al. 2009). In this case, the authors made use of a robust scalar coupling network between the 3-fluoro ^{19}F, Cε, Cδ, and Hδ, nuclei in the form of an HCCF 2D (or 3D) NMR experiment. The Cδ, and Hδ chemical shifts were separately connected to the Cβ, Hβ chemical shift pairs (Yamazaki et al. 1993), which were in turn correlated with backbone resonances via an HNCACB experiment.

In the current study, the fluoroaromatic probe used (3-FPhe) is not ^{13}C, or ^{15}N enriched, precluding the above approach. However, the fluorine nucleus is scalar coupled to the δ, ζ, and ε aromatic protons, with magnitudes in the 6–10 Hz range, as shown in Fig. 1c. The available scalar coupling network can be used to connect ^{19}F nuclei to the adjacent δ proton via an INEPT transfer, while this δ proton resonance can in turn be connected to the β, α and HN nuclei using ^{19}F, and ^{15}N-edited ^{1}H, ^{1}H NOESY experiments. The importance of the delta proton to this strategy precludes the use of 2-FPhe, while a moderately larger ($\sim$10%) ^{1}H-^{19}F coupling was the main advantage of the 3-FPhe over 4-FPhe. The efficiencies of INEPT and NOE based magnetization transfers must be taken into consideration and it is for this reason that we have chosen to demonstrate our assignment strategy using Calmodulin (CaM), a medium sized protein whose backbone and sidechain assignments have been thoroughly characterized.

Calmodulin is a ubiquitous calcium sensor protein which binds, and activates a variety of enzymes involved in cell signaling pathways (Hoeflich and Ikura 2002). Upon coordination of calcium, the protein undergoes a well-characterized conformational transition exposing hydrophobic surfaces which mediate binding to a variety of target proteins, hormones and peptides (Crivici and Ikura 1995). CaM is a 148 residue, acidic protein composed of two structurally analogous domains (N and C-terminal) connected by a flexible linker (residues 73–83) (Barbato et al. 1992). Each domain has two calcium binding EF-hand motifs composed of two helices (E and F) flanking a 12-residue loop which coordinates calcium with pentagonal bipyramidal symmetry (Malmendal et al. 1999). There are eight phenylalanine residues in *Xenopus laevis* calmodulin, whose locations are indicated on the X-ray structure in Fig. 1a. These include five phenylalanines in

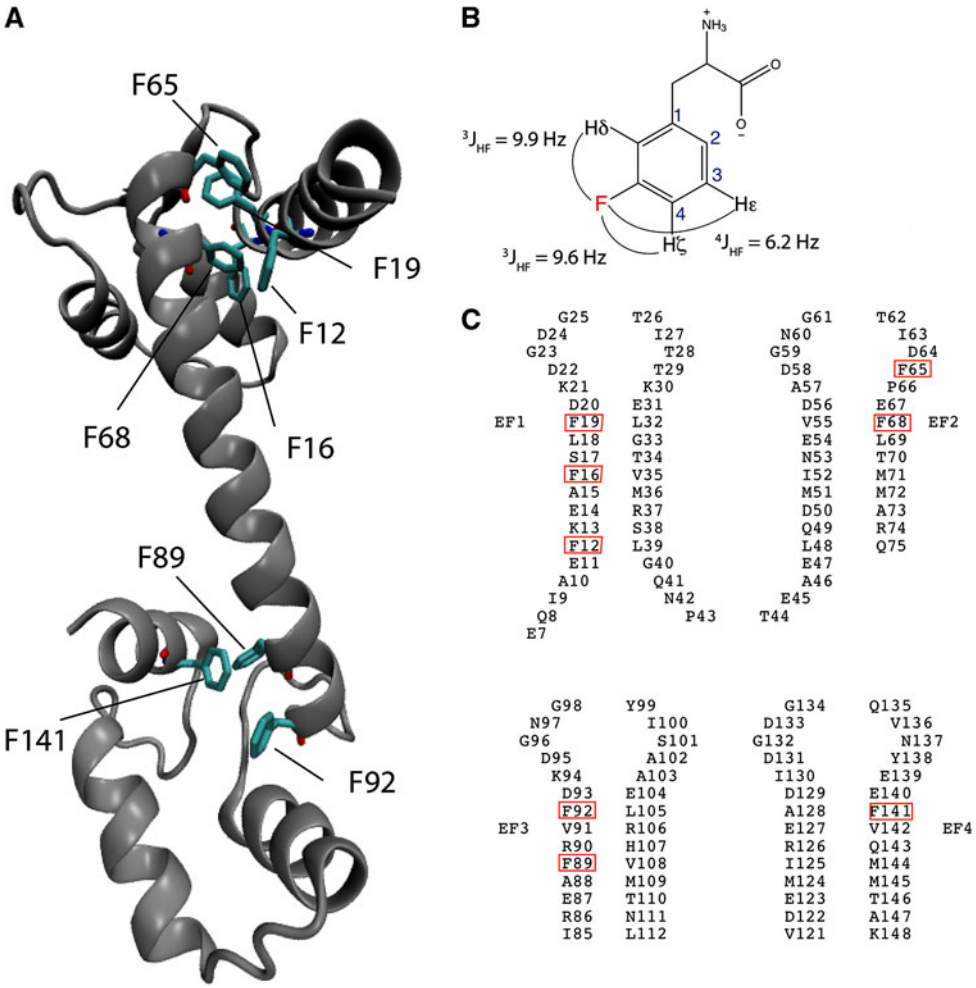

Fig. 1 **a** X-ray structure of calmodulin (PDB file 1CLL) showing the location of the eight phenylalanine residues. **b** 3-fluorophenylalanine structure with relevant scalar couplings indicated. **c** Secondary structural map of residues within the four EF hands of *Xenopus laevis* calmodulin

the N-terminal domain (residues 1–72) F12, F16, F19, F65 and F68; and three in the C-terminal domain (residues 84–148) F89, F92 and F141. Phenylalanines of CaM are highly conserved among species, and have been shown to contribute to the stability of the hydrophobic core as well as target binding and activation (Okano et al. 1998). Phenylalanine 92, specifically, has been shown to play a pivotal role in the conformational change associated with the apo to holo transition of the C-terminal domain such that mutation of this residue to alanine results in reduced or complete loss of enzyme activation (Meyer et al. 1996).

We begin with the careful analysis of structural perturbations resulting from the incorporation of 3-fluorophenylalanine by comparing ^{1}H-^{15}N HSQC NMR spectra and calcium binding constants of CaM in the presence and absence of the fluorine probe. We then asses the difficulties of resonance assignment using the direct replacement of phenylalanine residues with tyrosine by site-directed mutagenesis, which in our case provided only one assignment. Finally, we conclude with the details of the assignment of our eight fluorine resonances using a series of ^{19}F- and ^{15}N-edited NMR experiments.

Results

Assessment of structural perturbations

To evaluate structural and functional consequences of 3-fluorophenylalanine incorporation we rely on ^{1}H-^{15}N HSQC NMR spectra, and the measurement of calcium binding constants. Gross structural perturbations are routinely identified by preparing uniformly ^{15}N-enriched protein and subsequently comparing ^{1}H-^{15}N HSQC NMR spectra of a protein with and without fluorine labeling. Figure 2 shows an overlay of ^{1}H-^{15}N spectra of CaM with, and without incorporation of 3-fluorophenylalanine. Note that the phenylalanine resonances are completely absent from the 3-fluorophenylalaine labeled spectrum, indicating an enrichment of at least 95% based on the standard deviation of the noise. ^{1}H-^{15}N HSQC spectra of 3-FPhe CaM were indirectly assigned using previously published assignments for non-fluorinated calmodulin (Torizawa et al. 2004). Residues adjacent to 3-FPhe's were confirmed using ^{1}H-^{15}N NOESY-HSQC spectra (as well as ^{1}H NOESY spectra) which established correlations between these

residues and their '$n + 1$' neighbors. Although the chemical shifts of both fluorinated and non-fluorinated CaM are similar, there are measurable perturbations in residues near the eight phenylalanine sites, as well as considerable differences in line widths, suggesting that packing and dynamics of the backbone have been altered by fluorine incorporation. The relative stability of unlabeled and 3-FPhe labeled CaM was characterized using ^{1}H-^{15}N HSQC NMR spectra collected as a function of temperature between 37 and 80°C. The melting temperature of both proteins were estimated by monitoring the intensities of a subset of well-resolved peaks, and found to be approximately 76 and 73°C for unlabeled and 3-FPhe labeled CaM, respectively. Due to the high thermal stability of calmodulin and temperature limitations of the NMR probe, we were unable to fully characterize the unfolding and we emphasize that these values are estimates. However, it is clear that uniform labeling with 3-FPhe has not caused a significant decrease in the thermal stability.

It is also possible to assess structural perturbations from the perspective of calcium affinity, for both non-fluorinated and 3-FPhe labeled CaM. The binding constants for non-fluorinated and 3-FPhe CaM are $K_1 = 2.4 \times 10^5\ M^{-1}$, $K_2 = 1.1 \times 10^6\ M^{-1}$, $K_3 = 3.8 \times 10^4\ M^{-1}$, $K_4 = 1.2 \times 10^5\ M^{-1}$ and $K_1 = 2.6 \times 10^5\ M^{-1}$, $K_2 = 1.5 \times 10^6\ M^{-1}$, $K_3 = 8.6 \times 10^4\ M^{-1}$, $K_4 = 1.7 \times 10^5\ M^{-1}$ respectively. The minor difference in K_3, which represents a calcium coordination site in the N-terminal domain, may be due specifically to F65, which occupies position 10 of the calcium binding loop of EF-hand 2 (Fig. 1c). Alternatively, this may simply be a cumulative effect arising from the addition of five ^{19}F species in the hydrophobic core of the N-terminal domain as opposed to only three such species in the C-terminal domain. Overall, these values agree fairly well with those previously published (Linse et al. 1991), leading us to conclude that calcium binding in the presence of 3-fluorophenylalanine is effectively unperturbed under the current experimental conditions.

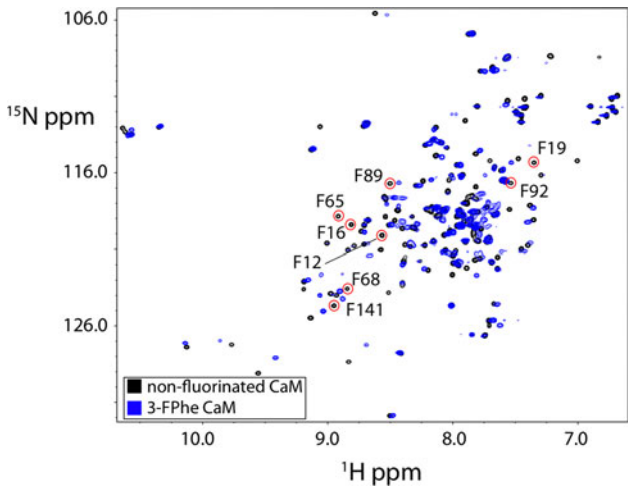

Fig. 2 ^{1}H-^{15}N HSQC spectra of 3-fluorophenylalanine labeled calmodulin overlaid with an analogous spectrum of non-fluorinated calmodulin

Site-directed mutagenesis

Single phenylalanine to tyrosine mutants were prepared for residues 12, 16, 19, 65 and 89 of CaM. The ^{19}F NMR spectra of each mutant, aligned with the wild type spectrum, are shown in Fig. 3. There are six resolved resonances in the wild type spectrum, with the most downfield peak integrating to roughly three times the intensity of the others. The relatively small chemical shift dispersion is perhaps not surprising considering that the N- and C-terminal domains of CaM each consist of two, predominantly helical, EF-hand motifs as shown in Fig. 1a. Several pairs of phenylalanine residues occupy identical positions within the EF-hand motif (Fig. 1c). For example, residues F89 and F16, F68 and F141 as well as F92 and F19, which are likely to experience similar environments. The spectra of the single phenylalanine to tyrosine mutants all exhibit substantial chemical shift perturbations (See Fig. 3). Although the substitution of a phenylalanine residue to a tyrosine is the most natural choice among the available standard amino acids, there is a substantial change in both size and polarity, which is perhaps magnified when considering residues within the protein core. The average increase in volume for a buried phenylalanine to a buried tyrosine has

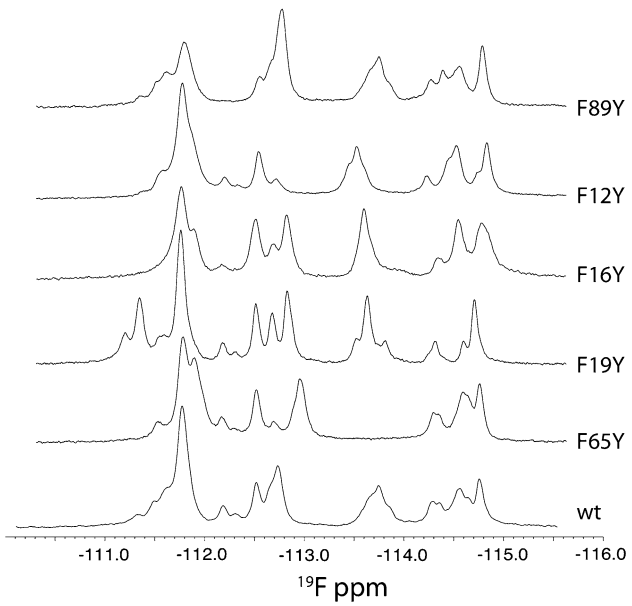

Fig. 3 ^{19}F NMR spectra of wild type (wt) and single phenylalanine to tyrosine mutants of calmodulin uniformly labeled with 3-fluorophenylalanine. All spectra were run under identical experimental conditions, as described in the "Materials and methods" section

been estimated to be only 0.2 Å^3 (Richards 1977). Considering the high density of native protein cores, where packing efficiency often exceeds 75% and is generally higher for α-helical structures (Woolfson 2001), this small increase in volume has the potential to cause significant disruptions in the geometric arrangement of core residues. The burial of polar groups in the non-polar interior of a protein is known to be destabilizing due to a large enthalpy of dehydration; however, this effect can be compensated for if the polar group forms a non-native hydrogen bond (Loladze et al. 2002). In general, mutations involving the rearrangement of hydrophobic core interactions reduce the so-called conformational uniqueness of a protein which has the potential to affect both structure and function (Willis et al. 2000). In addition, the above effects are likely to be amplified in situations where the residues under consideration are clustered in regions which share van der Waals contacts (Drake et al. 1993). Considering the complex interplay of effects, it is likely that the success of mutagenesis approaches is both probe, and protein dependant.

The ^{19}F NMR spectra of the single phenylalanine to tyrosine mutants of CaM reveal significant variation in chemical shift, line width, and minor conformers, as shown in Fig. 3. While it is possible the Phe to Tyr substitutions have altered the calcium affinity, it is doubtful that under the experimental conditions (9:1 $CaCl_2$:CaM) there is a significant population of protein in the apo form. A marked decrease in calcium affinity has been observed in previous studies of a E140Q C-terminal calmodulin mutant (Evenas et al. 1997), but unlike residue 140 which is a calcium coordinating ligand, none of the phenylalanine residues are involved in calcium coordination, or, apart from F65, located in the calcium binding loops.

In an effort to evaluate the relative perturbations introduced by the single replacement mutations we considered ^{19}F NMR difference spectra between the wild type and mutant species. After normalizing the wild type spectrum to 8, and the single mutant spectra to 7, the integral of the difference spectrum is expected to be close to 1, assuming global perturbations are minimal. In all cases, the difference spectra gave integrals of ∼4 suggesting that the perturbations are global; making spectral assignment using single replacement mutants impractical. ^{1}H-^{19}F HSQC NMR spectra of single mutant samples were collected in an attempt to improve resolution by relying on both ^{19}F and ^{1}H chemical shifts. Unfortunately, several residues have coincident or very similar aromatic proton chemical shifts, which combined with the overlap and perturbations in the ^{19}F dimension provides no additional assignment information. With the exception of F65Y, these perturbations prohibit conclusive assignment of the fluorine resonances. Some preliminary assignments may be gleaned from F89Y and F16Y, where it is clear that in both cases the intensity of the most downfield peak is attenuated. These observations combined with recognizing the identical positioning of these two residues in the EF-hand motif (Fig. 1c) allow for a tentative assignment of this resonance to F89 and F16. The fact that significant perturbations are observed in several of the single mutants is not surprising as, particularly for the N-terminus, the phenylalanine residues are clustered together within the core of the domain and any disruption in one position is likely to affect the others through van der Waals interactions. It is clear that complete, unambiguous assignments cannot be established using site-directed mutagenesis alone.

NMR-based assignment strategy

In the absence of ^{13}C-enriched 3-fluorophenylalanine, one can establish a connectivity between the ^{19}F probe and backbone nuclei by relying on scalar couplings between the fluorine nucleus and the ε, ζ, and the adjacent δ aromatic protons. The δ aromatic proton can then be correlated to the backbone resonances using NOESY-based magnetization transfer as described below. The intraresidue coupling between the ^{19}F nucleus and the adjacent δ proton is clearly delineated via a ^{1}H-^{19}F HSQC, as shown in Fig. 4a. A ^{19}F 1D NMR spectrum is displayed along the indirect axis for clarity. For each fluorine resonance, the ε, ζ, and the adjacent δ proton are resolved due to the similarly sized scalar coupling between these nuclei and the fluorine atom (see Fig. 1b). Note that we also attempted to establish such a correlation via a ^{1}H-^{19}F heteronuclear NOE (Rinaldi 1983); however, despite the small couplings, the HSQC proved to exhibit the greatest fidelity. The next step in the assignment protocol involves connecting the δ proton to the intraresidue β protons using a ^{1}H-^{19}F NOESY-HSQC, which was collected as a 2D spectrum as shown in Fig. 4b. Due to the relatively few fluorine-coupled δ protons and their generous chemical shift dispersion, the ^{19}F dimension was not collected, but simply used to edit the spectrum. The observed crosspeaks were compared with those in a ^{1}H,^{1}H NOESY spectrum, which aided in the verification of the final assignments. As an example of the assignment protocol, we trace the connectivity network between the ^{19}F resonance of F92 (−114.68 ppm) and the amide proton of the adjacent residue, aspartic acid 93. Beginning with the chemical shift of the fluorine coupled δ proton (7.13 ppm) we find a crosspeak between this resonance and a β proton whose chemical shift is 2.59 ppm, as shown in Fig. 4b. This β proton resonance is then correlated to the backbone using a ^{1}H-^{15}N NOESY-HSQC experiment, where assignments of the ^{1}H-^{15}N plane are either transferred from previously published data, or obtained experimentally using a sample uniformly enriched in ^{15}N, and ^{13}C. Because the fluorinated phenylalanine probes are not

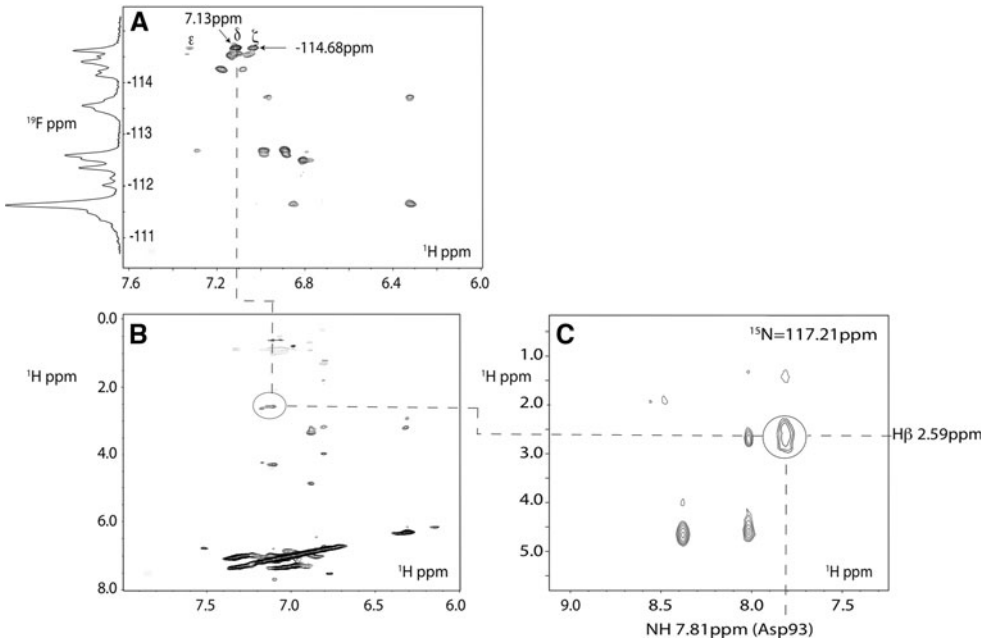

Fig. 4 NMR-based assignment strategy for phenylalanine 92. **a** ^{1}H-^{19}F HSQC spectrum with ^{19}F 1D trace along the indirect detect fluorine axis. The ε, δ, and ζ proton crosspeaks are indicated for F92. **b** ^{1}H-^{19}F NOESY-HSQC spectrum. The experiment was collected as a 2D (i.e. without evolving the ^{19}F dimension) and shown are the first indirect and direct detect dimensions. The crosspeak highlighted corresponds to the beta protons of F92. **c** ^{1}H-^{15}N NOESY-HSQC spectrum. All three dimensions of the experiment were collected. Shown is the nitrogen plane corresponding to 117.21 ppm. The crosspeak indicated corresponds to the amide proton of aspartic acid 93. All experiments were run at 37°C. Additional experimental details can be found in the "Materials and methods" section

^{15}N enriched, the β protons were linked to the amide proton and nitrogen chemical shifts of the neighboring, $n + 1$ residue. Continuing with the assignment of the example resonance, a distinct crosspeak is observed between a β proton at 2.59 ppm and an amide proton with a chemical shift of 7.81 ppm in the nitrogen plane corresponding to 117.21 ppm, as shown in Fig. 4c. The $n + 1$ residue was identified as aspartic acid 93 from previously published assignments (Torizawa et al. 2004); confirming that the associated fluorine resonance arises from phenylalanine 92. The above steps were repeated for the remaining resonances with details provided in the supplementary material. Of the eight residues, there were four crosspeaks observed in the ^{1}H-^{19}F NOESY-HSQC, resulting in the assignments of phenylalanine residues 68, 89, 92 and 141. While the strategy is quite straightforward, the methodology is limited by the dependence on dipolar magnetization transfer, which is highly influenced by dynamics. The ^{19}F 1D NMR spectra of 3-FPhe labeled CaM (see Fig. 3) reveal a significant variation in resonance line widths as well as the presence of minor conformers, indicating the prevalence of chemical exchange over a range of dynamic timescales, which may reduce the magnitude of NOESY-based magnetization transfer for several residues. The weak NOEs in combination with the relatively small scalar couplings ($J_{HF} = 9.9$ Hz) available for INEPT transfers are likely factors in the reduced number of crosspeaks observed in the ^{1}H-^{19}F NOESY-HSQC. Apart from phenylalanine 68, the crosspeaks obtained all come from the C-terminal domain of CaM. It is possible that the dynamics of the N-terminal domain are such that the NOE transfers are weak, and therefore unobservable within a reasonable amount of experimental time.

Trypsin fragments of calmodulin

To clarify resonance assignments, we separately examined ^{19}F NMR spectra of the N-terminal and C-terminal domains of calmodulin upon proteolytic cleavage in the linker region. Controlled digestion of calmodulin in the presence of calcium with trypsin produces two fragments (Drabikowski et al. 1977), commonly referred to TR1C and TR2C, composed of the N-terminal (residues 1–73) and C-terminal (84–148) lobes, respectively. The resulting fragments retain identical secondary and tertiary structural features of the full length protein (Finn et al. 1995; Ishida et al. 2000) as well as calcium binding affinity (Linse et al. 1991), but exhibit reduced or absent activation ability on target proteins (Walsh et al. 1977). An overlay of ^{1}H-^{15}N HSQC spectra of 3-FPhe TR1C and 3-FPhe TR2C with non-fluorinated calmodulin are in good agreement with respect to the chemical shifts, as shown in Figs. 5a, b. ^{19}F NMR spectra of TR1C and TR2C are shown in Fig. 6, aligned with an analogous spectrum of the full length

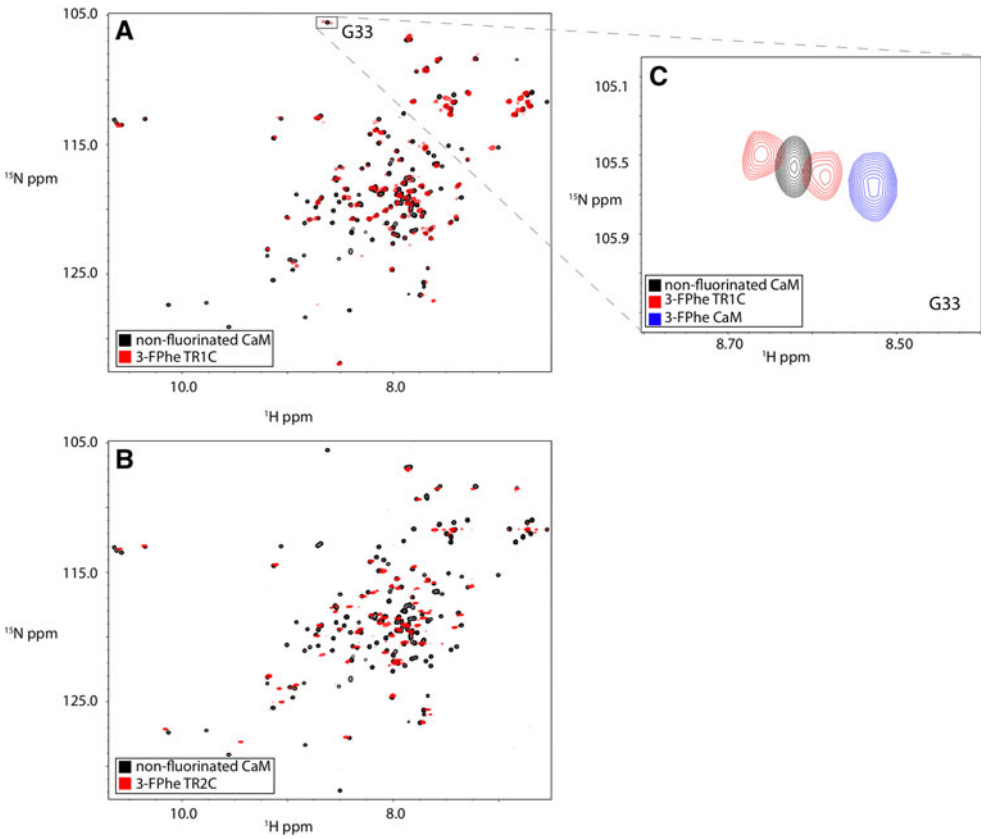

Fig. 5 ^{1}H-^{15}N HSQC spectra of 3-FPhe TR1C (**a**) and 3-FPhe TR2C (**b**) overlaid with an analogous spectrum of non-fluorinated calmodulin. The indicated region of the spectrum enclosing glycine 33 is magnified in (**c**) with the corresponding region from an ^{1}H-^{15}N HSQC of 3FPhe CaM shown in *blue*

protein. The ^{19}F NMR spectra of TR1C and TR2C have five and three major resonances resolved respectively, in addition to some minor peaks which may represent secondary conformations in slow exchange on the ^{19}F NMR timescale. An interesting feature of the fragment spectra is the increased resolution in TR1C of the two downfield peaks. These two peaks, along with the most downfield peak in TR2C have coincident chemical shifts in the full length protein. The discrepancy between the fluorine chemical shifts in the full and TR1C spectra suggest that there is an interaction between the two domains in the context of the full length protein which affects the local environment of the fluorine probes. Careful examination of the ^{1}H-^{15}N HSQC spectra of both 3-FPhe TR1C and full length CaM reveal two distinct conformations with close to equal populations for a number of residues, as shown clearly for glycine 33 in Fig. 5c. The two conformations appear to correspond to slow exchange between native-like states, as evidenced by the similarity of the chemical shifts for G33 in TR1C and non-fluorinated CaM, as compared to 3-FPhe CaM (see Fig. 5d). There is precedence in the literature for large scale fluctuations about a flexible region of the long interconnecting helix (Baber et al. 2001; Bertini et al. 2004), as well as a recent crystal structure of a closed, globular form of the protein where the two domains are within contact (Fallon and Quiocho 2003). It is likely that

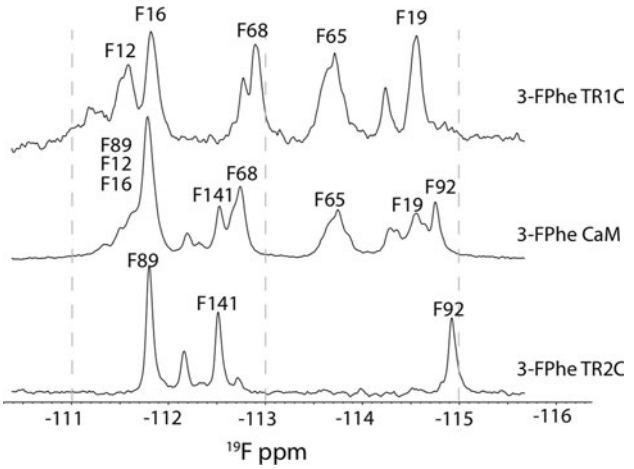

Fig. 6 Assigned ^{19}F NMR spectra of uniformly 3-FPhe labeled TR1C, CaM and TR2C

in solution the classic barbell topology and this more recent collapsed, globular structure represent two possible conformational extremes which can be accessed in solution, while the presence of 3-fluorophenylalanine may alter the energetics of the system, and thus the conformational equilibrium in favor of a species where the two terminal domains interact. A more complete investigation of the observed disparity in the ^{19}F and ^{1}H-^{15}N NMR spectra of

TR1C and the full length protein is currently being investigated.

The NMR based assignment strategy, utilizing ^{1}H-^{19}F HSQCs, ^{1}H-^{19}F NOESY-HSQCs and ^{1}H-^{15}N NOESY-HSQCs, was repeated on TR1C (data provided in supplementary material), and full assignments were obtained and transferred to the full length protein spectrum. The fully assigned ^{19}F NMR spectra of TR1C, TR2C and 3FPhe-CaM are shown in Fig. 6.

Conclusions and final remarks

In this paper we have described an NMR based strategy for the assignment of resonances associated with mono-fluorinated aromatic amino acid probes in proteins, in the absence of sidechain ^{13}C and ^{15}N enrichment. We take advantage of the prominent scalar couplings between the ^{19}F probe and the aromatic protons to correlate the fluorine nucleus to the ε, ζ, and the adjacent δ protons via the ^{1}H-^{19}F HSQC. Fluorine coupled δ protons are then connected to intraresidue β protons using a ^{1}H-^{19}F NOESY-HSQC. In the final step, the β protons are associated with the $n + 1$ residue amide and nitrogen chemical shifts to complete the assignment via a ^{1}H-^{15}N NOESY-HSQC. In principle the basic steps applied here can be used to assign fluorinated aliphatic residues and other unnatural amino acids by adjusting experimental parameters for the NMR active nucleus at hand. The dependence of this assignment strategy on NOESY-based magnetization transfer makes it particularly sensitive to dynamics and additional relaxation mechanisms, such as chemical exchange and chemical shift anisotropy (CSA) common to fluoraromatics, which reduce the magnitude of the NOE effect.

Discrepancies between the ^{19}F and ^{1}H-^{15}N chemical shifts of TR1C with that of the full length are an indication of a conformational exchange process related to interdomain interactions. This is particularly interesting in light of previous reports indicating large-scale fluctuations about the flexible linker and the existence of transient domain–domain interactions. The minor conformers observed in both the full-length protein and the trypsin fragments reflect motions such as ring flipping or conformational reorganization, which are slow on the chemical shift timescale. Attempts to investigate these properties using ^{19}F T_1 and CPMG T_2 experiments at several temperatures and field strengths were performed. However, control experiments using fractionally fluorinated CaM revealed that these intermediate and slow motions were exclusive to the fully 3-FPhe labeled CaM. While the assignment protocol outlined above is robustly applicable to any scenario wherein the protein is ^{19}F- and ^{15}N-labeled, the details of the structural and dynamic characteristics of fully and fractionally ^{19}F labeled CaM will be outlined in a forthcoming manuscript.

Materials and methods

Expression and purification of uniformly ^{15}N-enriched 3-fluorophenylalanine labeled CaM

Incorporation of *DL*-3-fluorophenylalanine via heterologous expression (Evanics et al. 2007) as well as purification of CaM (Ikura et al. 1990) was performed as previously described with slight modifications. A plasmid (pET21b) encoding *Xenopus laevis* calmodulin (residues 1–148) was transfected into BL21(DE3) under control of the T7 promoter. LB broth inoculated with a single colony was grown overnight and used to inoculate 1 L of M9 minimal media supplemented with 0.3% D-glucose, 0.1% $^{15}NHCl_4$, 100 mg/L ampicillin, 10 mg/L thiamine, 10 mg/L biotin, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$. Uniform labeling with 3-fluorophenylalanine was achieved by first allowing cell cultures at 37°C to reach an OD_{600} of 0.8, whereupon 1 g/L glyphosate, 75 mg/L *DL*-tryptophan, and 75 mg/L *DL*-tyrosine is added. Once cell cultures reached an OD_{600} of 1.0 (after approximately 1 h), 35 mg/L *DL*-3-fluorophenylalanine was added and expression was induced with the addition of 238 mg/L IPTG. Cell cultures were harvested after 3.5 h by centrifugation at 7,000 rpm for 20 min. Cells were then resuspended in 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF buffer at pH 8 and lysed by incubation at 4°C in the presence of 1 mg/mL lysozyme for 30 min, followed by sonication. After the addition of DNase (10 μg/mL) and RNase (5 μg/mL), the suspension was centrifuged at 9,000 rpm for 20 min at 4°C and the cleared lysate was purified using Ni–NTA Agarose resin (Qiagen, Mississauga, Ontario, Canada). The labeled protein was further purified using phenyl Sepharose as described previously (Ikura et al. 1990). Aliquots containing CaM were pooled, concentrated and exchanged into 20 mM Bis-Tris, 0.1 M KCl, 9 mM $CaCl_2$, 0.2% NaN_3 buffer at pH 8 using centrifugal concentrators for subsequent NMR experiments. Calcium-free calmodulin samples were prepared as previously described (Zhang et al. 1995) for the determination of macroscopic calcium binding constants. All buffers used in the preparation of calcium-free CaM were made with water which had been decalcified using Chelex-100 resin and stored in plastic bottles which had been treated with 5 mM ethylenediaminetetraacetic acid (EDTA), followed by extensive rinsing with decalcified water. EDTA was added to purified, dilute protein samples ($\sim$200–500 μM) to a final concentration of 20 mM, followed by precipitation with trichloroacetic acid (TCA). The sample was redissolved in 25 mM

NH_4HCO_3 and passed through a Sephadex G-25 column. Collected protein was then exchanged into 20 mM Bis-Tris, 0.1 M KCl, 2 mM EDTA, 0.2% NaN_3 buffer at pH 7.5.

Site-directed mutagenesis

Single phenylalanine to tyrosine mutations were prepared for phenylalanine residues 12, 16, 19, 65 and 89. Site-directed mutagenesis was performed using the QuikChange Multi protocol (Stratagene, Cedar Creek, TX). Plasmid sequences were verified by automated DNA sequencing (SequeTech, Mountain View, CA).

Trypsin digest of CaM and 3-fluorophenylalanine CaM

Limited trypsin digestion of full length CaM and 3-fluorophenylalanine CaM was carried out as previously described (Drabikowski et al. 1977). Trypsin (TPCK treated from bovine pancreas; 10,000–13,000 BAEE U/mg) and soybean trypsin inhibitor (STI—10,000 BAEE U/mg) were purchased from Sigma–Aldrich (Oakville, Ontario, Canada) and used without further purification. In a typical digestion, 1 mM CaM was dissolved in 50 mM NH_4HCO_3, 50 mM NaCl, 5 mM $CaCl_2$ at pH 7.9 and then equilibrated at 37°C for 1 h with 0.017 mM trypsin. Digestion was quenched by the addition of 0.017 mM soybean trypsin inhibitor on ice. The purification was carried out as detailed previously (Brokx and Vogel 2002). All NMR samples were first concentrated, then buffer exchanged into 20 mM BIS-Tris, 0.1 M KCl, 9 mM $CaCl_2$, 0.2% NaN_3 buffer at pH 8.

Determination of macroscopic binding constants

Macroscopic calcium binding constants were measured using a competitive chelator assay as described previously (Linse et al. 1991). 5,5′-Br_2-BAPTA was obtained from Molecular Probes (Eugene, Oregon). Calmodulin and 3-fluorophenylalanine labeled calmodulin were decalcified as described above and titrated with 1 mM $CaCl_2$ in the presence of 5,5′-Br_2-BAPTA in 20 mM Bis-Tris, 0.1 M KCl, at pH 8, prepared with decalcified water at 25°C. The titration was monitored using UV spectroscopy until the $OD_{263\ nm}$ indicated saturation. The raw data was fit using Caligator software (Andre and Linse 2002) to obtain K_1 through K_4.

NMR experiments

NMR experiments were performed on a 600 MHz Varian Inova spectrometer (Varian Inc., Walnut Creek, CA). For direct observe ^{19}F NMR experiments and all ^{15}N, ^{1}H HSQC correlation experiments, a 5 mm HCN/FCN triple resonance single gradient salt-tolerant cryogenic probe, tunable to either ^{1}H or ^{19}F, was used with typical pulse lengths for ^{19}F, ^{1}H, and ^{15}N of 9.5, 9.25, and 39 μs respectively. ^{1}H-^{19}F HSQC and ^{1}H-^{19}F NOESY-HSQC experiments were performed on a 5 mm HFCN quad probe (Varian Inc., Walnut Creek, CA) capable of simultaneous high power pulses on both ^{1}H and ^{19}F. Typical pulse widths for ^{19}F and ^{1}H used were 9 and 8.5 μs respectively. All protein sample concentrations were 1–1.5 mM in 90% H_2O/10% D_2O 20 mM Bis-Tris, 0.1 M KCl, 9 mM $CaCl_2$ 0.2% NaN_3 at pH 8, and experiments were run at 37°C. ^{19}F 1D NMR experiments were collected with 1,024 transients using a spectral width of 8,000 Hz and a 1.5 s recycle delay for 3-fluorophenylalanine labeled CaM. ^{1}H-^{19}F HSQC experiments were modified from ^{1}H-^{15}N HSQC pulse programs from Biopack software (Varian Inc.) and were typically collected with 256 transients and 96 increments spanning 3,200 Hz in the indirect dimension. ^{1}H-^{19}F NOESY-HSQC experiments were modified from the corresponding ^{1}H-^{15}N NOESY-HSQC pulse programs from Biopack (Varian Inc) and were collected as 2D experiments in 2,048 transients with 64 increments spanning 5,800 Hz in the first indirect dimension using a 400 ms mixing time. ^{1}H-^{15}N NOESY-HSQC experiments from Biopack (Varian Inc.) were collected in 32 transients with 32 and 64 increments spanning 8,000 and 2,400 Hz in the first and second indirect dimensions, respectively. ^{1}H Watergate NOESY spectra were collected in 64 transients with 256 increments spanning 6,500 Hz in the indirect dimension using a 200 ms mixing time. ^{1}H-^{15}N HSQC pulse sequences were obtained from Biopack software (Varian Inc.) and typically were collected in 4 scans with 80 increments spanning 2,000 Hz in the indirect dimension. Chemical shifts were referenced using the Varian 'setref' macro, which in the absence of a standard, establishes the ^{1}H, ^{15}N, and ^{19}F chemical shifts indirectly from the lock signal (Harris et al. 2002). All NMR data were processed using NMRPipe/NMRDraw (Delaglio et al. 1995), and analyzed with NMRView software (Johnson and Blevins 1994).

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