Effects of Phe-to-Trp mutation and fluorotryptophan incorporation on the solution structure of cardiac troponin C, and analysis of its suitability as a potential probe for in situ NMR studies

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Abstract

^{19}F NMR spectroscopy is potentially a powerful tool for probing protein properties in situ. However, results obtained using this technique are relevant only if the ^{19}F probe offers minimal perturbation to the surrounding environment. In this paper, we examine the effect of 5-fluorotryptophan (5fW) incorporation on the three-dimensional structure of cardiac troponin-C (cTnC), with the intention of developing a ^{19}F -labeled TnC for use in in situ ^{19}F NMR. We find that, in general, 5fW does not perturb the structure of the protein significantly. Replacement of residue Phe 153 with 5fW produces no noticeable change in protein conformation. However, replacement of residue Phe 104 with 5fW produces a folding behavior that is dependent on the *Escherichia coli* strain used to express the mutant. The orientations of the indole rings in these mutants are such that the Trp residue adopts a χ^2 of $\sim 90^\circ$ in the F104W mutant and $\sim -100^\circ$ in the F153W mutant. Using results from ^{19}F - ^1H heteronuclear NOE experiment, we show the replacement of L-Trp with 5fW at these positions does not change the orientation of the indole ring and the spread of the 5fW side-chain dihedral angles increases moderately for the F104(5fW) mutant and not at all for the F153(5fW) mutant. Based on these structures, we conclude that the substitution of Phe by 5fW at these two positions has minimal effects on the structure of cTnC and that the 5fW indole rings in both mutants have well-defined orientation, making the two mutants viable candidates for use in in situ ^{19}F NMR spectroscopy.

Keywords: protein stability and mutagenesis; solution NMR; structure and folding

^{19}F NMR spectroscopy is a powerful tool for probing dynamic and conformational variations in macromolecules (Hull and Sykes 1974, 1975, 1976; Sixl et al. 1990;

Eccleston et al. 1993; Campos-Olivas et al. 2002). Besides being helpful in investigating proteins in solution, ^{19}F NMR can also be an effective technique for in situ investigations. A few strategically placed ^{19}F atoms in a large macromolecular assembly can reveal valuable information regarding the orientation and the motions of proteins in the complex (Grage et al. 2002; Ulrich 2005).

Fluorine holds several advantages over other nuclei commonly used in NMR. Its inherent absence in most natural organisms reduces its background noise in NMR spectra. The high gyromagnetic ratio of this spin one-half nucleus coupled with 100% natural abundance means the detection of fluorine signal is much easier than those of ^{13}C or ^{15}N atoms. The electronic structure of the fluorine atom gives

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Abbreviations: Tn, troponin; (c, s)TnC, (cardiac, skeletal) troponin C; (c, s)TnI, (cardiac, skeletal) troponin I; 5fW, 5-fluorotryptophan; 4fW, 4-fluorotryptophan; 6fW, 6-fluorotryptophan; F104W, Phe 104 to Trp; F153W, Phe 153 to Trp; F104(5fW), Phe 104 to 5fW; F153(5fW), Phe 153 to 5fW; NOE, nuclear Overhauser enhancement; CSA, chemical shift anisotropy.

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the atom a wide chemical shift spread, making its resonance frequency far more sensitive to its magnetic environment than most other atoms, a valuable asset for probes of protein orientation and dynamics. However, the wide CSA of the atom also induces faster transverse relaxation in solution, reducing its sensitivity in many situations.

Many methods are used to fluorine label the protein of interest (Sykes and Weiner 1980) and biosynthetic incorporation of fluorinated amino acids are among the most popular schemes used. Despite the widespread application of these fluorinated amino acids, very few systematic studies have been conducted to investigate fluorine's perturbation on protein structures. In most investigations involving fluorine-labeled proteins, perturbation due to fluorine is considered minimal or inconsequential. However, considering fluorine is known to alter chemical properties of compounds, fluorination cannot be inert in every situation. Since it is impossible to predict how fluorine is going to change a protein's structure or affect a protein's biological function, it is essential that we build up semiempirical knowledge on the perturbation to protein structure due to insertion of a fluorine atom into an environment of a specific character, e.g., hydrophobic pocket or charged ligand binding site.

Pratt and Ho (1975) and Browne et al. (1970) have independently examined the relative toxicity of fluorotryptophans on bacteria. Both found that 4-fluorotryptophan (4fW) supported bacterial growth better compared to 5- (5fW) or 6-fluorotryptophan (6fW) (see Fig. 1B for an illustration of the indole ring carbon numbering system and proton nomenclature). They also examined the specific activity of fluorotryptophan-containing β -galactosidase and found, once again, 4fW-containing isomers of the enzyme have far higher activity compared to 5fW and 6fW. However, 5fW continues to be widely used in the community (Campos-Olivas et al.

2002; Salopek-Sondi et al. 2003; Abbott et al. 2004; Anderluh et al. 2005). Xiao and coworkers (Xiao et al. 1998) investigated conformational changes induced by global substitution of Tyr by 3-fluorotyrosine in glutathione transferase using X-ray crystallography. While substitution of the active site residue Tyr 6 reduced the catalytic efficiency of the enzyme by 10-fold owing to both the change in the pK_a of the aromatic OH group and steric clashes between the fluorine and the substrate, most other mutations produced only minor changes in the protein conformation (Xiao et al. 1998). A number of other studies (both theoretical and experimental) have demonstrated that in some proteins perturbations from ^{19}F are minimal (Lau and Gerig 1997; Campos-Olivas et al. 2002). A notable observation is that a polar ^{19}F atom can sit comfortably inside a hydrophobic pocket without disturbing the hydrophobic core packing (Campos-Olivas et al. 2002).

In this paper, we studied the effects of fluorinated amino acid 5fW on the structure of human cardiac troponin C, a classic Ca^{2+} -binding EF-hand protein responsible for regulating muscle contraction. Our motive for studying the ^{19}F -labeled cTnC comes from a desire to investigate the mechanism of TnC-mediated muscle regulation. With a ^{19}F -labeled cTnC, solid-state NMR techniques can be applied to obtain orientation information for cTnC embedded within myofibrils (Ulrich 2005) and the knowledge of the orientational dynamics of cTnC during muscle activity will be valuable to the construction of the muscle regulatory model. To engineer such a ^{19}F -labeled TnC isoform, we utilized the lack of Trp in wild-type TnC to construct two Phe-to-Trp mutants that can be labeled with fluorotryptophans. This approach allows a single label to be present on each molecule, reducing spectral complexity and simplifying data analysis. Phe residues are chosen over Tyr residues mainly because the Tyr residues in TnC are solvent-exposed and too flexible to correlate well with the orientation of the domains.

Most EF-hand motifs follow the consensus sequence of $h_1**h_2(x)(y)*(z)*G(-y)I(-x)**(-z)h_3**h_4$, where x , y , z , $-x$, $-y$, and $-z$ represent Ca^{2+} -chelating amino acids, $*$ represents any amino acids; and h represents hydrophobic amino acids. For the C-terminal domain of cTnC, h_1 and h_2 of the first EF-hand motif, as well as h_3 and h_4 of the second EF-hand motif are Phe. Our decision was to mutate the h_2 of the first EF-hand motif, which is residue 104, and the h_3 of the second EF-hand motif, which is residue 153, to Trp (Fig. 1A). By putting the mutations on two nonparallel helices near two different Ca^{2+} -binding loops, we reduce the chance of protein misfolding induced by having two mutations on the same helix. The skeletal troponin C equivalents of both mutants containing L-Trp have been studied before, and the mutations are found to have minimal effects on the biological function of the protein. A study

Figure 1. (A) Ribbon representation of the C-terminal domain of the wild-type chicken cTnC. Side chains of F104 and F153 (sites of mutation) are labeled red and blue, respectively. (B) Indole ring of Tryptophan. Numbering of the carbon is illustrated. Each proton is labeled using nomenclature used in the paper. In 5fW, $\text{H}\zeta 3$ is replaced with a ^{19}F atom.

done on F105W mutant of skeletal TnC (the skeletal analog of the F104W mutant) by Trigo-Gonzalez reasoned, from an observed red shift in the absorbance spectrum of the F105W mutant, that Ca^{2+} -binding caused the tryptophan ring to become more buried (Trigo-Gonzalez et al. 1992). However, no changes in Ca^{2+} -binding affinity of the C-terminal domain was observed by them. Chandra and coworkers (Chandra et al. 1994) did a thorough study on the sTnC analog of the F153W mutant. They observed a twofold decrease in the Ca^{2+} affinity of the mutant, which they attributed to the destabilization of the hydrophobic pocket packing of sTnC by the bulky side chains of tryptophan. However, physiological assays of the mutant using reconstituted Tn-tropomyosin-actomyosin ATPase complex showed the mutant produced the same ATPase activity as the wild type, both in the presence and absence of Ca^{2+} , indicating the change in the mutant's Ca^{2+} affinity is not enough to reduce biological activity (Chandra et al. 1994). In this study, we closely scrutinized the effects of Phe-to-Trp substitutions as well as 5fW incorporation on the structure of cTnC. Using NMR techniques, we determined the high resolution structures of all four isoforms of the protein, putting the emphasis on the structure of the C-terminal domain where the substitutions reside. The orientation of the indole ring in each isoform was also carefully determined using data from homonuclear NOE as well as ^{19}F - ^1H heteronuclear NOE experiments.

Results

Expression of 5fW-labeled mutants

In this study, both BL21 and W3110TrpA33 strains were used to express fluorotryptophan-containing mutants. In the case of BL21, glyphosate was used to inhibit aromatic amino acid synthesis and exogenous sources of Phe, Tyr, and Trp were used to supplement the media. Owing to the toxic nature of 5fW, the culture was grown to an approx OD_{600} of 1.0 before 150–200 mg of the racemic mixture of D- and L-5fW was added. However, when the BL21-expressed apo F104(5fW) mutant was titrated with Ca^{2+} , it became obvious that the Ca^{2+} affinity of the protein's C-terminal domain was reduced relative to its N-terminal domain, and the most likely cause was 5fW-induced misfolding during the transition from apo to Ca^{2+} -saturated state. Normally, because the C-terminal domain of TnC has much higher Ca^{2+} affinity compared to the N-terminal domain, wild-type TnC becomes Ca^{2+} -saturated in the C-terminal domain before significant Ca^{2+} -binding is detected in the N-terminal domain (Li et al. 2002). This is exemplified by the NMR-monitored Ca^{2+} titration of the F104W

mutant presented in Figure 2A. However, in the BL21-expressed F104(5fW) mutant the N-terminal domain binds Ca^{2+} before the C-terminal domain. This is indicated by the migration of the N-terminal domain G70 amide proton signal upon Ca^{2+} addition before the signal corresponding to amide proton of residue G146 in the Ca^{2+} -saturated C-terminal domain begins to grow in intensity (Fig. 2B). It was also noted that the final intensities of most signals from the Ca^{2+} -saturated C-terminal domain were only half the size of their counterparts from the N-terminal domain. This is in contrast to cTnC isoforms that fold normally. In those cases, signals from the Ca^{2+} -saturated C-terminal domain are the same size as those from the N-terminal domain (Fig. 2A). This indicates that not only is the Ca^{2+} affinity of the C-terminal domain reduced but a portion of the C-terminal domain is also not folding properly. This hypothesis was further confirmed by 1D ^{19}F spectra taken during the titration, which showed that besides the main peak at -49.1 ppm, multiple signals between -49.6 ppm and -50.0 ppm can be seen at the end of the titration, indicating that many conformational states of the protein exist (Fig. 2C). To ensure the abnormality was not caused by the mutation from Phe to Trp, we performed Ca^{2+} titrations on mutants containing L-Trp. Both F153W and F104W mutants showed a normal pattern of Ca^{2+} binding (a representative set of data from the Ca^{2+} titration of the F104W mutant is shown in Fig. 2A). Even though the $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC NMR spectra of these Phe-to-Trp mutants in their Ca^{2+} -saturated state are not identical to that of the wild-type cTnC (Fig. 3), the patterns of chemical shift changes upon metal binding are very similar to the wild type and the presence of minor populations or misfolded proteins are not observed. Originally, we thought the observed reduction in the Ca^{2+} affinity and misfolding we saw in the F104(5fW) mutant's C-terminal domain was a consequence of 5fW incorporation. However, the same mutant expressed in *Escherichia coli* Trp auxotrophic strain W3110TrpA33 exhibited normal Ca^{2+} binding patterns (data not shown); thus, the phenomenon we observed seems to be restricted to the F104(5fW) mutant produced in BL21 cells.

Several hypotheses can be formulated to explain these observations. The 5fW could be modified by enzymes expressed specifically by BL21 but not by W3110TrpA33, leading to decreased Ca^{2+} affinity of the C-terminal domain. However, there is yet no experimental evidence to support this claim. Mass spectrometry of the samples showed that BL21-expressed F104(5fW) mutant sample contained a 14-kDa degradation product of TnC, whereas the proteins produced by W3110TrpA33 are intact. Because the 14-kDa product elutes at the same time as the intact protein in reverse-phase HPLC, the amount of the

Figure 2. Ca^{2+} -titrations of Phe-104 mutants. (A) 1D traces from $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC spectra taken during the Ca^{2+} titrations of the F104W mutant. (B) 1D traces from $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC spectra taken during the Ca^{2+} titrations of the BL21-expressed F104(5fW) mutant. (C) 1D ^{19}F spectra taken during the Ca^{2+} titration of the BL21-expressed F104(5fW) mutant. In A and B, the strip on the left is the amide proton of residue 146 and the strip on the right is the amide proton of residue 70. In the case of the F104W mutant titration, the C-terminal domain was Ca^{2+} -saturated before the N-terminal domain. But in the case of BL21-expressed F104(5fW), the trend is the opposite. Results from the Ca^{2+} titrations of the wild-type cTnC and the F153W mutant are very similar to that of the F104W mutant (data not shown). In C, note the two broad signals between -49.6 ppm and -50 ppm.

degradation product present in the sample cannot be quantified. However, the size of the MS signal from the 14 kDa product is 70% of that from the intact protein; therefore, its amount in the sample is significant. In view of these facts and the observation that W3110TrpA33 grew much more robustly in the presence of 5fW than BL21, we think that 5fW caused far more toxic effects in BL21 cells than in W3110TrpA33 cells, leading to the production of higher amount of proteases in BL21. Since the C-terminal domain of TnC is unstructured in its apo state, proteolytic cleavage

likely occurred in the C-terminal domain, hampering its ability to bind Ca^{2+} and leading to decreased signal intensities from residues in the Ca^{2+} -saturated C-terminal domain.

Effects of ^{19}F incorporation on backbone amide proton/nitrogen chemical shifts

In many situations, the replacement of a proton by a ^{19}F atom is considered a conservative substitution. Presence

Figure 3. $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC spectra of (A) wild-type cTnC, (B) the F104W mutant, (C) the F153W mutant, (D) the F104(5fW) mutant, and (E) the F153(5fW) mutant. Peak patterns in all five HSQC spectra are very similar. Signals from some residues exhibiting signal doubling in the $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC spectrum of the F153(5fW) mutant are labeled in C and E.

of only a single ^{19}F atom often changes little in the way of steric and electrostatic interactions in the region. However ^{19}F does possess a different electronic configuration than a proton. Under the right circumstances, this can induce a change in the local magnetic environment, which can manifest itself as changes in the chemical shifts of nuclei surrounding it. The HSQC spectrum of the F104(5fW) mutant largely resembles that of the F104W mutant (Fig. 3C,E). However, many residues in the F153(5fW) mutant give two signals in the $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC (Fig. 3E). All of these residues are found in the C-terminal domain of the protein. Each pair of signals consists of a strong peak and a weak peak, with the weak peak having exactly the same chemical shifts as a peak in the F153W mutant $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC spectrum. Besides residue 153 itself, other residues exhibiting discernable signal doubling included residues 98, 100, 119, 121, 136, 137, 148, and 154 to 158. All the residues are mapped to a region close to the location of the ^{19}F atom (Fig. 4). Analysis of the heteronuclear spectral data also showed that the signals in each pair have the same neighboring residues as well as identical side chain atom chemical shifts and amide proton NOE patterns; thus, they must represent the same residue. Based on these observations, we think these signal doublings are a manifestation of the incomplete 5fW-labeling of the protein. For residues

close to the indole ring, the amide nitrogen and amide proton chemical shifts are sensitive enough to detect the difference between a ^{19}F atom and a proton. The fact that signal doubling was not seen with the F104(5fW) mutant means that either the ^{19}F incorporation is more complete for the F104(5fW) mutant, or the side chain of 5fW in the F104(5fW) mutant is not close enough to any residue to produce a significant change in the chemical shifts of amide protons and nitrogens. However, even in the case of the F153(5fW) mutant, not every residue

Figure 4. Visual representation of the ^{19}F -induced signal doubling in $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC of the F153(5fW) mutant. Residues exhibiting the signal doubling phenomenon are colored red on the ribbon representation of the backbone structure of the F153(5fW) mutant. The ^{19}F atom is shown as an orange sphere. All residues showing signal doubling are found in the vicinity of the ^{19}F atom.

close to the ^{19}F atom exhibited signal doubling. In particular, residues 112 and 117 showed no change in their amide nitrogen/proton chemical shifts. No signal doubling was observed in any atom other than the amide nitrogen and amide proton. This is expected, since amide nitrogens and amide protons are known to be much more sensitive to the environment than the side chain atoms.

Structures of the mutants

To evaluate the effects of the mutations and the 5fW-incorporation on the structure of TnC, we determined the solution structures of all four mutants using NMR techniques, and compared them to the structure of the wild-type chicken cTnC (PDB code 1AJ4) determined by Sia et al. (1997). To solve the structures, conventional methodology used in multidimensional protein NMR was followed. In particular, chemical shift assignments of the backbone atoms (N, C_α , HN) were first established using data from the HNCACB (Wittekind and Mueller 1993) and CBCACONNH (Grzesiek and Bax 1992) experiments. Chemical shifts of the side chain ^{13}C and ^1H were then assigned using data from the HCCHTOCSY (Sattler et al. 1995), HCCONH (Lyons and Montelione 1993), and CCONH (Grzesiek et al. 1993) experiments. Once the proton chemical shifts were known unambiguously, data from 3D aliphatic and aromatic ^{13}C -edited NOESY as well as 3D ^{15}N -edited NOESY experiments were automatically analyzed using CYANA. Distance and dihedral restraints derived from the data were then used to generate structures using both CYANA and Xplor-NIH. For more details on this procedure, please consult the experimental section.

Of all the structures solved in this study, none had a significantly different N-terminal domain structure compared to the wild-type chicken cTnC. This is not surprising, since the N-terminal domain sequence of the mutants (residues 1–90) shares 100% identity with the

N-terminal domain sequence of wild-type chicken cTnC. Together with the fact that the two domains of cTnC do not interact in solution, substitutions in the C-terminal domain should have no effect on the N-terminal domain. Therefore, the following analysis on the structures of the mutants will focus entirely on the C-terminal domain, with the emphasis on the orientations of the indole ring in each structure.

Structures of F104W and F104(5fW) mutants

The Phe-to-Trp mutation at position 104 resulted in a change of ~ 2.5 ppm and 2.6 ppm in the residue's amide nitrogen and α carbon chemical shifts, respectively (1.5 ppm and 2.2 ppm after correcting for the change in residue type and assuming random structure [Wishart et al. 1991]) compared to the wild-type cTnC. The replacement of L-Trp with 5fW in the mutant did not induce additional perturbation judging by the small amide proton/nitrogen chemical shift differences between the F104W and the F104(5fW) mutants. The only significant amide nitrogen chemical shift changes induced by the incorporation of 5fW occurred to a few residues on the F helix (residues 119, 120, 121, and 124). This is not surprising, since the side chains of these residues are very close to atom H ζ 3 of the indole ring. However, no significant chemical shift changes were seen with any other atoms in these residues.

In this study CYANA was used to automatically assign and calibrate the signals in all 3D NOESY spectra. For the F104W mutant, CYANA was able to find a total of 1545 unambiguous distance restraints with 183 of them long-range restraints. Structure calculation carried out using these restraints and the dihedral angle restraints derived using TALOS revealed that the structure of the F104W mutant is very similar to that of the wild-type cTnC (Fig. 5A). The 20 lowest energy structures from a total of 50 structures calculated superimposed with a backbone RMSD of 0.80 ± 0.14 Å while the backbone of the well-defined region superimposed

Figure 5. Ensembles of backbone structures of (A) the F104W mutant, (B) the F104(5fW) mutant, (C) the F153W mutant, and (D) the F153(5fW) mutant. Each ensemble contains the superimposition of 20 lowest energy structures from the 50 calculated. Only the structured portion of the C-terminal domain (residues 95–158) is shown in each ensemble.

Table 1. Structural statistics for the C-terminal domain structures of F104W, F104(5fW), F153W, and F153(5fW) mutants

Isoform	F104W	F104(5fW)	F153W	F153(5fW)
NOE restraints				
Total	1545	1272	2111	1709
Intraresidue	683	497	695	570
Sequential	424	409	637	534
Medium-range	255	264	496	389
Long-range	183	122	273	216
Backbone dihedral restraints				
φ	98	119	91	106
ψ	101	116	86	106
Restraints violations				
Distance > 0.1 Å	8	11	5	7
Dihedral > 1°	0	0	0	0
RMSD to average structure				
Well-defined regions ^a	0.62 ± 0.11 Å	0.61 ± 0.12 Å	0.62 ± 0.12 Å	0.60 ± 0.17 Å
All regions ^b	0.80 ± 0.14 Å	0.94 ± 0.15 Å	0.82 ± 0.10 Å	0.91 ± 0.22 Å
φ ψ angle stats ^c				
Percentage in most favored region	89.8	89.4	91.7	89.9
Percentage in additional allowed region	8.0	8.6	7.0	7.9

^a Using residues in the range of 95–158 with backbone RMSD < 1.00.

^b Using all residues in the range of 95–158.

^c Using residues in the range of 95–158 as determined by the program Procheck.

with an RMSD of 0.62 ± 0.11 Å (see Table 1 for definition of well-defined region). A total of 89.8% of all backbone dihedral angles are in the most favored region of the Ramachandran map (Table 1). The major difference between the wild-type cTnC and the F104W mutant lies in the interhelical angles between helices E and F, as well as helices G and H, with the F104W mutant being slightly more “open” compared to the wild type (Table 2; Fig. 6A). The situation for the F104(5fW) mutant is similar. CYANA found 1272 unambiguous distance restraints for this protein, the least of all mutants. Out of all the restraints, 122 are long-range restraints. Despite the smaller number of restraints, the backbone RMSD of 20 lowest energy structures superimposed is 0.94 ± 0.15 Å, with the well-defined region having an RMSD of 0.61 ± 0.12 Å, almost as good as the F104W structure. In fact, the structure of the F104(5fW) mutant has slightly better agreement with the wild-type cTnC than does the F104W mutant (Fig. 6B).

For both the F104W and the F104(5fW) mutants, the hydrophobic packing around the indole ring is not significantly disturbed. This is most likely because the side chain of residue 104 is not deeply buried in the hydrophobic core. It lies above the hydrophobic core, with fewer packing constraints compared to the side chain of residue 153. In the F104 mutant structures, variations in the orientation of the indole ring are mostly determined by changes in the χ^2 angle for the Trp residue. From the 3D aromatic ^{13}C -edited NOESY data, a number of unambiguous distance restraints involving indole ring protons were obtained (Fig. 7A). Structure calculations

showed all restraints are consistent with each other and a single, well-defined orientation of the indole ring, with a side-chain χ^2 dihedral angle of $\sim 96^\circ$ (Table 3), was able to satisfy all the restraints. The orientation of the ring is similar but not identical to that of the wild-type F104 side chain. The H ζ 3 atom is pointed down toward the F helix while the H ϵ 1 points toward the side chains of residues 97 and 100. Interestingly, even if no distance restraints are used for any indole ring protons but atoms H ζ 3 and H ϵ 1, the ring orientation is still well defined and agrees with the ring orientation obtained with a full set of restraints. This fact turned out to be relevant in defining the 5fW indole orientation in the F104(5fW) mutant. Owing to the lack of ^{13}C -labeling on the ring, we can only obtain NOE data for the fluorine and the H ϵ 1 proton on the indole ring. Even though signal intensities in the FHOESY experiment are lower compared to the 3D ^{13}C -edited NOESY experiment, a combination of the directly detected ^1H dimension and lack

Table 2. Interhelical angles for the C-terminal domains of wild-type and mutant cTnC

	E/F (°)	G/H (°)
Wild-type chicken cTnC ^a	115 ± 4	121 ± 4
F104W	82 ± 9	89 ± 11
F104(5fW)	106 ± 8	111 ± 6
F153W	108 ± 8	115 ± 5
F153(5fW)	92 ± 9	116 ± 6

^a PDB code 1AJ4.

Figure 6. Ribbon representations of backbone superimposition of the wild-type chicken cTnC structure (PDB code 1AJ4) with (A) the F104W structure (red), (B) the F104(5fW) structure (gray), (C) the F153W structure (orange), (D) the F153(5fW) structure (yellow). The wild-type structure is shown in blue in all superimpositions. RMSDs between the mutant structures and the wild-type structure are shown *below* each ribbon representation. In all panels, the superimposition is done using residues in structured regions only (residues 95–103, 111–123, 130–140, and 147–158).

of spectral overlap in the ^{19}F dimension means the FHOESY data has higher resolution and is easier to analyze and less ambiguous compared to the ^{13}C -edited NOESY data. The mixing time used in the FHOESY experiment is also kept short to minimize ^1H - ^1H spin diffusion artifacts in the experiment. Comparing the NOE contact pattern made by the proton $\text{H}\zeta_3$ on the indole ring with the ^{19}F atom's heteronuclear NOE contact pattern, we see little, if any, difference (Fig. 7B).

This is only possible if the orientation of the 5-fluoroindole ring in the F104(5fW) mutant is similar to that of the indole ring in the F104W mutant. Just as in the F104W case, specifying only distance restraints involving the ^{19}F and $\text{H}\epsilon_1$ is enough to produce a well-defined ring orientation that is very similar to that of the F104W mutant. In fact, although we were able to obtain the chemical shift assignments for protons $\text{H}\eta_2$ and $\text{H}\epsilon_3$ using the FHOESY experiment, adding distance restraints from these two

Figure 7. (A) Strips taken from the 3D aromatic ^{13}C -edited NOESY spectrum of the F104W mutant, illustrating the intramolecular contacts made by the indole ring protons. (B) Detailed comparison between the NOE contacts made by the $\text{H}\zeta_3$ proton on the Trp of the F104W mutant and the ^{19}F atom on the 5fW of the F104(5fW) mutant. The NOE contact patterns are virtually identical between the two. Although the intensities of heteronuclear NOE are weaker than that of the homonuclear NOEs, the resolution of the ^{19}F - ^1H heteronuclear NOE experiment is much higher, allowing to the identification of many unambiguous NOEs.

Table 3. Trp and 5fW side-chain dihedral angles of the mutants

	χ^1 (°)	χ^2 (°)
F104W	-67 ± 4	96 ± 6
F104(5fW)	-70 ± 14	83 ± 23
F153W	177 ± 4	-96 ± 3
F153(5fW)	-175 ± 6	-106 ± 6

atoms did not improve the definition of the 5-fluoroindole ring orientation.

Structure of F153W and F153(5fW) mutants

Compared to the F104W mutant, the mutation of Phe to Trp at position 153 produced no change in amide nitrogen chemical shift and a relatively small change in α carbon chemical shifts. Since there is no sign of secondary structure change at either position, the difference in mutation-induced chemical shift changes between the two mutation sites could be purely due to differences in dynamics or exposure to solvents at the two sites. The side chain of the F153 residue is a more integrated part of the hydrophobic core than that of F104. Replacing the Phe with the bulky Trp, and subsequently the bulkier 5fW, could be potentially disruptive to the overall packing of the core. However, the physiological study done by Chandra et al. (1994) showed that the mutant has the same physiological activity as the wild type, while the Ca^{2+} affinity is decreased only by half. Inspection of the $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC of either the F153W mutant or the F153(5fW) mutant showed that both have well-folded C-terminal domains.

The 3D aliphatic ^{13}C -edited NOESY spectrum of the F153W mutant was collected on the INOVA 800 MHz spectrometer. The sensitivity and high resolution of the spectrum are reflected in the large number of distance restraints CYANA was able to derive from the data. A total of 2111 unambiguous restraints were found, with 273 of them being long-range restraints, the most of all mutants. The 20 lowest energy structures, of a total of 50 calculated, superimposed with a backbone RMSD of 0.82 ± 0.10 Å, 0.62 ± 0.12 Å for the well-defined regions. Almost 92% of all backbone dihedral angles are found in the most favored region of the Ramachandran map. The interhelical angles between helices E and F, and helices G and H of the F153W mutant also agreed well with the wild-type C-terminal domain structure. The backbone RMSD between the F153W mutant and the wild-type C-terminal domain structure is only 0.94 Å for the well-defined regions. For the F153(5fW) mutant, a total of 1709 unambiguous distance restraints were found, of which 216 were long-range restraints. The RMSD between the 20 lowest energy structures (out of 50 calculated) after superimposition is 0.91 ± 0.22 Å. The well-defined regions in these structures

superimposed with an RMSD of 0.60 ± 0.17 Å. Just like the other mutants, almost 90% of all backbone dihedral angles are found in the most favored region of the Ramachandran map. There is a slight difference in the interhelical angle of helices E and F between the F153(5fW) mutant and the wild-type cTnC (Table 2), with the F153(5fW) mutant being slightly more “open” than the wild-type cTnC. As a result, the two structures superimposed with a slightly higher backbone RMSD for well-defined regions.

The hydrophobic packing of the F153W and the F153(5fW) mutants are little disturbed by the replacement of the phenyl ring with the bulkier indole ring. Superimposition of the C-terminal domain of the F153W mutant with the C-terminal domain of the wild-type protein revealed that the conformations of all the hydrophobic side chains in the core are in good agreement between the two structures. For the F153W mutant, the 3D aromatic ^{13}C NOESY allowed the extraction of close to 30 unambiguous NOEs for the six protons on the indole ring. Qualitative examination of each indole proton's NOE contact patterns alone was enough to define the orientation of the ring unequivocally (Fig. 8A). The orientation of the indole ring is such that the amide proton on the ring points away from the hydrophobic pocket and toward the E helix, making several contacts with the side chains of Leu 100 and Leu 97. The H ζ 3 on the indole ring points deep into the hydrophobic pocket, contacting side chains of the Ile residues in the middle of the short anti-parallel β -sheet that forms the back of the hydrophobic pocket. The χ^2 angle for the residue is $\sim -95^\circ$ (Table 3). The normal of the indole ring plane is roughly parallel to the helical axis of helix G.

The orientation of the F153(5fW) mutant's fluoroindole ring is determined using the NOE contacts involving only the ^{19}F atom and the amide proton. The NOE contact pattern made by the fluorine agreed well with that of the H ζ 3 proton in the F153W mutant (Fig. 8B). Subsequent structure refinements done using the distance restraints from the heteronuclear experiment revealed an orientation of the fluoroindole ring that is almost identical to the orientation of the indole ring in the F153W mutant.

Owing to the larger number of restraints that were found for the ^{19}F atom, the fluoroindole ring orientation of the F153(5fW) mutant is much better defined compared to the F104(5fW) mutant. The average value of the χ^2 angle for the 5fW is $\sim -106^\circ$ with a standard deviation of only 6° (Table 3).

Discussion

Expression of 5fW-labeled proteins

Many studies have demonstrated that using nonauxotrophic *E. coli* strains to produce fluorotryptophan-

Figure 8. (A) Strips taken from the 3D aromatic ^{13}C -edited NOESY spectrum of the F153W mutant, illustrating the intra-molecular contacts made by the indole ring protons. (B) Detailed comparison between the NOE contacts made by the $\text{H}\zeta 3$ proton on the Trp of the F153W mutant and the ^{19}F atom on the 5fW of the F153(5fW) mutant. The NOE contact patterns are virtually identical between the two.

labeled proteins is possible provided that the endogenous Trp synthesis is shut down, either by overloading the medium with fluorotryptophan or through the use of enzyme inhibitor like glyphosate. However, this study demonstrated that there are disadvantages to this approach. Inhibition of the natural amino acid synthesis can induce the expression of other enzymes. These changes in bacterial metabolism can lead to degradation and modification of the protein of interest, leading to lower yield and modified protein properties. It is also important to screen for an *E. coli* strain with a high tolerance to the fluorinated amino acid being used. BL21, which is robust and adaptable in most scenarios, has very low tolerance for 5fW. The presence of 5fW at a concentration of 100 mg/L was enough to stop bacterial growth when $\text{OD}_{600} < 0.8$. At higher OD_{600} , although bacteria did continue to grow, the stressed cells produced higher levels of proteases that degraded the protein of interest. 5fW could also have triggered the expression of a number of metabolic enzymes in BL21, leading to the modifications of the fluorinated amino acid. All these factors might have contributed to the misfolding we saw with the BL21-expressed F104(5fW) mutant. The Trp auxotroph W3110TrpA33, on the other hand, was very tolerant of 5fW. It grew robustly in its presence and produced a much higher yield of protein than BL21.

Potential use of the mutants as probes in *in situ* NMR

As the determination of high-resolution protein structures becomes easier and information in structural biology begins to accumulate, many scientists have come to the realization that knowing the structure of a single protein is no longer adequate in many situations. Far more insight can be gained by understanding how proteins interact with each other. Thus, researchers are switching their focus to investigating the behavior of proteins in complex with other macromolecules in their *in situ* environment. This is especially true in the field of muscle regulation research, where muscle regulatory proteins exist only as a part of the thin filament. Owing to the sizes of these complexes, conventional structural biology techniques usually are not applicable. As a result, a new approach is often used to investigate these large complexes: Structures of proteins composing the complex are first solved individually, then by obtaining a few crucial pieces of information regarding their relative distances and orientations to each other in the complex, the structure of the entire complex is constructed (Corrie et al. 1999). This is also our strategy for investigating movements of TnC during muscle activation. Utilizing the orientation dependency of ^{19}F CSA tensor, information on the orientation of TnC on the filament during muscle contraction and relaxation can be elucidated if the orientation of the C–F bond

vector correlates well with the orientation of the protein. Our study showed that, in both mutants, the fluoroindole rings have well-defined orientations; therefore they should be acceptable probes for the orientation of TnC. The 5fW in the F104(5fW) mutant possessed an average χ^2 of $83 \pm 22^\circ$, while the 5fW in the F153(5fW) mutant had an average χ^2 of $-106 \pm 6^\circ$. The fact that the 5fW in the F104(5fW) mutant had larger χ^1 and χ^2 dihedral angle spreads than the F153(5fW) mutant could be a reflection of its more dynamic indole ring motions. Molecular dynamics simulations conducted for both mutants showed that the indole ring in the F104(5fW) mutant undergoes ring oscillations of considerably larger magnitude than that of the F153(5fW) mutant, which experiences almost no movement (X. Wang and B.D. Sykes, unpubl.). However, the motions experienced by the F104(5fW) indole ring are at a much higher frequency than the NMR timescale and thus will have no bearing on the results of the NMR experiments. Owing to the inherent degeneracy in the process of orientation determination using the CSA tensor, two or more CF bond vectors, all of which must have different orientation in the protein, will be needed to solve the protein's orientation. The CF bond vectors in the two mutants are at an average angle of 24° with each other. This implies, with the exceptions of a few situations, that the two vectors will reveal nondegenerate information, an important detail in the case where protein orientation was to be determined using axially symmetric chemical shift tensors.

Structures of the F104 and F153 mutants

In all mutants, the secondary structures are well preserved. The major difference between the mutant structures and the wild-type structure lies in the difference in interhelical angles. Determination of interhelical angles using NOE derived distance restraints alone is not always a robust process (Chou et al. 2001). The presence of one or two long-range restraints is enough to change the interhelical angle by 20° or more. For this reason, some ambiguous NOE signals from long-range contacts were manually assigned before applying the CYANA automatic assignment protocol. It is also evident from the $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC spectra as well as the presence of important long-range NOE signals in the 3D ^{13}C -edited NOESY spectra that all mutants are well-folded and adopt the "open" conformation of the wild-type cTnC C-terminal domain. Given the above information, we believe the differences in the interhelical angles between the F104W mutant and the wild-type structure, as well as that between the F153(5fW) mutant and the wild-type structure, are not significant enough to be an indicator

of major structural change in the mutants. Since the amount of solvent-accessible hydrophobic surface area is a reliable measure of the degree of "openness" in cTnC (Sia et al. 1997), we also examined sizes of solvent-accessible hydrophobic surface area on the C-terminal domain of each mutant structure using the program VADAR (Willard et al. 2003). For all mutants, the average amount of solvent-exposed hydrophobic area is not significantly different than that of the wild type, indicating no significant change in the openness of the structure is induced by Trp or 5fW.

Signal doubling in the F153(5fW) mutant

Fluorotryptophan incorporation in a protein is never 100%, even using the most carefully controlled protocols. The exact percentage of incorporation can be estimated using a number of methods (Campos-Olivas et al. 2002; Valencia et al. 2003). Our study shows that the $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC can be used as a measure of the fluorine incorporation efficiency. In the case of the F153(5fW) mutant, we were able to calculate the ratio between the intensities of the peak produced by the fluorinated isoform and the peak produced by the nonfluorinated isoform. Of the 12 residues showing the signal-doubling phenomenon, 10 residues had clear and nonoverlapping peaks. For all 10 peaks, the intensity ratio of the strong peaks and the weak peaks was approximately 4:1. This implies that about 80% of protein is 5fW-labeled, a number that agrees well with the estimation done using fluorescence measurements.

Materials and methods

Site-directed mutagenesis

(C35S, C84S) human cTnC cDNA (Pearlstone et al. 2000) was used as the template in the construction of the F104W and F153W mutants. The F104W mutant was constructed using the method of site-directed mutagenesis by overlap primer extension (Ho et al. 1989). Primers 5'-CCCGCGCCCCGCCA TATGGATGACATCTACAAGGCT-3' (cTnC-5') and 5'-TT TGTCCACATGCGGAAGAGGTC-3' (F104-3') were used to clone one-half of the mutant cDNA while primers 5'-TGAC CTCTTCCGCATGTGGGACAAA-3' (F104-5') and 5'-ACG ACAGTGGATCCTCAGCATCTACTCCACAC-3' (cTnC-3') were used to clone the second half of the cDNA. The two halves were subsequently connected by performing PCR using the products from the first round as templates and primers cTnC-5' and cTnC-3' as extending primers. The full-length mutant cDNA was then digested with NdeI and BamHI and ligated into pET3a fragments that were precut with NdeI and BamHI. The F153W mutant was produced by PCR using primers cTnC-5' and 5'-ACGACAGTGGATCCCTACTCCACACCCCTCA TGAATCCAGCCACTCATCATAG-3' (F153-3') and wild-type cDNA as a template. In all cases the underlined bases in

the primer sequences are the mismatching bases that introduced the mutation into the protein. All primers were synthesized by the DNA core facility at Department of Biochemistry, University of Alberta.

Expression of the protein and incorporation of fluorotryptophan into F153W and F104W mutants

In this study, a combination of unlabeled and $^{15}\text{N}/^{13}\text{C}$ -labeled proteins containing either L-tryptophan or 5fW was used. Proteins containing normal tryptophan were expressed in *E. coli* strain BL21(DE3)pLysS using the procedure described previously (Sia et al. 1997). Incorporation of 5fW into BL21 expressed proteins was promoted by first inhibiting the aromatic amino acid synthesis using 1 g/L glyphosate, an inhibitor of the 5-enolpyruvylshikimate acid-3-phosphate synthase (Steinrücken and Amrhein 1980; Comai et al. 1983). The needed aromatic amino acids were then supplemented exogenously. When the culture reached an OD_{600} of 0.8–1.0, 150 mg of D,L-5fW (Sigma, F0896) was added. Half an hour later, the culture was induced using 120 mg/L IPTG. The induction was carried out for 3 h before harvesting the culture. To achieve a high fluorine incorporation efficiency, 5fW-containing mutants were also expressed in *E. coli* Trp auxotroph strain W3110TrpA33 that was infected with phage DE3 and transformed with plasmid pLysS (Valencia et al. 2003). In this procedure, 1 L of minimal M9 medium containing 100 mg of L-Trp was seeded with 10 mL of overnight culture grown in LB at 37°C and grown at the same temperature. When the cells reached an OD_{600} of ~1.0, they were spun down at 4°C for 10 min with a 4400g centrifugal force and the pellet was resuspended in fresh M9 media containing no Trp and grown for 30 min at 37°C; 150 mg/L of D,L-5fW was then added at the end of the 30 min. The cells were grown for another 30 min before induction was initiated with 120 mg/L of IPTG. The culture was harvested 3 h after the induction.

Purification of the mutants

The harvested cells were French pressed and the supernatant was applied to a DEAE-Sephadex (A25) column equilibrated with a buffer of 50 mM Tris, 0.1 M NaCl (pH 8.0). The column was washed extensively with the equilibration buffer at a flow rate of 0.6 mL/min before the protein was eluted with a 0.1–0.45 M NaCl salt gradient. The fractions containing the expressed proteins were pooled and lyophilized and redissolved in approximately 30 mL of a buffer containing 5 mM CaCl_2 , 50 mM KCl, 50 mM Tris, and 1 mM MgCl_2 (pH 7.5). The sample solution was loaded onto a phenyl-Sepharose column equilibrated with the same buffer. The column was washed with the same buffer for 5–8 h at a flow rate of 0.6 mL/min before an elution buffer containing 50 mM Tris and 1 mM EDTA (pH 7.5) was applied. The intact TnC was eluted as a single sharp peak.

Sample preparation and calcium titrations

All samples used for data collection contained 1.0–1.5 mM of protein, 100 mM KCl, 10 mM imidazole, 1.5 mM DSS, 10% D_2O , and 0.05% NaN_3 (pH 6.7). If a Ca^{2+} -saturated sample was required, CaCl_2 stock solution was added until Ca^{2+} concentration had reached 10 mM. To evaluate ^{19}F 's effect on the protein, qualitative Ca^{2+} titrations of the protein were performed as follows: Either 0.5 μL or 1 μL aliquot of 100 mM

CaCl_2 was added to a 500- μL sample containing ~1 mM of apo protein (pH 6.7), and 100 mM KCl, 10 mM imidazole, 0.2 mM DSS. The titration was monitored with either $\{^1\text{H}, ^{15}\text{N}\}$ -HSQC or one-dimensional ^{19}F spectroscopy, or both. The titration was stopped when the chemical shift and the line shape of the signal stopped changing.

Data acquisition and analysis

All data were acquired either on a Varian Inova 500 or an Inova 600 spectrometer at 30°C except the 3D aliphatic ^{13}C -edited NOESY for the F153W, which was collected on an Inova 800 spectrometer equipped with a 5-mm triple resonance probe with triple axis gradients. The 500- and 600-MHz spectrometers are equipped with 5-mm triple resonance probes with Z-axis pulse field gradients. With the exception of the ^{19}F - ^1H heteronuclear NOE experiment, all pulse sequences were obtained from the BioPack pulse sequence package distributed by Varian Inc. A mixing time of 75 msec was used for all 3D NOESY experiments used to derive distance restraints for structure calculations. All data were processed using the software package NMRPipe (Delaglio et al. 1995) and analyzed using NMRView (Johnson and Blevins 1994). Backbone assignments were done using data from HNCO, HNCACB (Wittekind and Mueller 1993), and CBCACONNH (Grzesiek and Bax 1992) spectra. The software package Smartnotebook (Slupsky et al. 2003) was used to expedite the process. Since most secondary structures in cTnC are α -helices, d_{NN} contacts from the 3D ^{15}N -edited NOESY experiment were also used to establish sequential assignments along the backbone. Side-chain assignments were done using data from conventional HCCHTOCSY (Sattler et al. 1995), HCCONH (Lyons and Montelione 1993), and CCONH (Grzesiek et al. 1993) experiments. The β -methylene protons and the methyl groups on Val and Leu in the mutants were not stereospecifically assigned.

Structural determination

All proton distance restraints used in determining structures were derived from 3D aliphatic and aromatic ^{13}C -edited NOESY and ^{15}N edited NOESY automatically using CYANA version 2 (Güntert 2004). NMRView was first used to automatically generate a peak list for each NOESY spectrum. The lists were then manually edited to remove spectral artefacts and noise. Some unambiguous peaks representing long-range contacts were also assigned manually before the lists were supplied to CYANA. Dihedral angle restraints obtained using TALOS (Cornilescu et al. 1999) were also included in the calculation. The automatic NOE assignments in CYANA were carried out using a tolerance of 0.025 ppm for the directly detected dimension, 0.04 ppm for the indirectly detected proton dimension, and 0.5 ppm for the ^{13}C or ^{15}N dimension. No distance restraints were allowed to be shorter than 1.8 Å or longer than 6 Å. Distance calibration in CYANA was done using a simple but robust scheme: Peaks with median intensity were assumed to represent a distance of 3.1 Å. Distance restraints from peaks with other intensities were extrapolated from this standard using the R^6 distance relationship commonly used for calibrating NOE distances. Twenty-four crystal structure-derived distance restraints between Ca^{2+} ions and chelating side chains were also used in the structure calculation. The automatic NOE assignment module in CYANA used an eight-round iterative structure refinement protocol in

which 100 random structures were calculated in the first round with no restraints applied and 50 structures were calculated for each successive round using NOE assignments based on previous round's structures. After eight rounds, 20 minimum energy structures were chosen from the 50 calculated as the final ensemble. Because the two globular domains of cTnC were known to be independent of each other, the default NOE assignment script was modified to eliminate any possible NOE assignments connecting C-terminal domain atoms with N-terminal domain atoms. The automatic backbone dihedral angle estimation feature of CYANA was not used, since experimentally determined backbone dihedral angles were available. The set of CYANA-optimized structures was further refined using a water-refinement protocol (Linge et al. 2003) implemented under the software package Xplor-NIH (Schwieters et al. 2003). For CYANA calculations, the force field parameter and topology for 5fW were defined exactly the same way as those for L-Trp. The force field parameter and topology for 5fW in Xplor-NIH calculations were adapted from those for L-Trp by changing atom type for atom HZ3 to fluorine and modifying the charges on HZ3 and CZ3 to -0.25 and 0.25 , respectively. Bond angles and bond lengths, as well as improper dihedral angles concerning these two atoms were not changed.

^{19}F - ^1H heteronuclear NOE determination

The distance restraints between ^{19}F and ^1H were determined using data from a ^{19}F - ^1H heteronuclear NOESY experiment (FHOESY), whose pulse sequence was adapted from a pulse sequence designed to measure heteronuclear NOEs (Rinaldi 1983; Kover and Batta 1987) for use with ^{19}F . In particular, ^{19}F was the indirectly detected nucleus and ^1H the observed nucleus. No ^{19}F decoupling was applied during the acquisition. Mixing times of 150 msec and 75 msec were used for the F104(5fW) and the F153(5fW) mutants, respectively.

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References

- Abbott, G.L., Blouse, G.E., Perron, M.J., Shore, J.D., Luck, L.A., and Szabo, A.G. 2004. ^{19}F NMR studies of plasminogen activator inhibitor-1. *Biochemistry* **43**: 1507–1519.
- Anderluh, G., Razpotnik, A., Podlesek, Z., Macek, P., Separovic, F., and Norton, R.S. 2005. Interaction of the eukaryotic pore-forming cytolysin equinatoxin II with model membranes: ^{19}F NMR studies. *J. Mol. Biol.* **347**: 27–39.
- Browne, D.R., Kenyon, G.L., and Hegeman, G.D. 1970. Incorporation of monofluorotryptophans into protein during the growth of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **39**: 13–19.
- Campos-Olivas, R., Aziz, R., Helms, G.L., Evans, J.N., and Gronenborn, A.M. 2002. Placement of ^{19}F into the center of GB1: Effects on structure and stability. *FEBS Lett.* **517**: 55–60.
- Chandra, M., McCubbin, W.D., Oikawa, K., Kay, C.M., and Smillie, L.B. 1994. Ca^{2+} , Mg^{2+} , and troponin I inhibitory peptide binding to a Phe-154 to Trp mutant of chicken skeletal muscle troponin C. *Biochemistry* **33**: 2961–2969.
- Chou, J.J., Li, S., Klee, C.B., and Bax, A. 2001. Solution structure of Ca^{2+} -calmodulin reveals flexible hand-like properties of its domains. *Nat. Struct. Biol.* **8**: 990–997.
- Comai, L., Sen, L.C., and Stalker, D.M. 1983. An altered *aroA* gene-product confers resistance to the herbicide glyphosate. *Science* **221**: 370–371.
- Cornilescu, G., Delaglio, F., and Bax, A. 1999. Protein backbone angle restraints from searching a database for chemical shift and sequence homology. *J. Biomol. NMR* **13**: 289–302.
- Corrie, J.E., Brandmeier, B.D., Ferguson, R.E., Trentham, D.R., Kendrick-Jones, J., Hopkins, S.C., van der Heide, U.A., Goldman, Y.E., Sabido-David, C., Dale, R.E., et al. 1999. Dynamic measurement of myosin light-chain-domain tilt and twist in muscle contraction. *Nature* **400**: 425–430.
- Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J., and Bax, A. 1995. NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6**: 277–293.
- Eccleston, J.F., Molloy, D.P., Hinds, M.G., King, R.W., and Feeney, J. 1993. Conformational differences between complexes of elongation factor Tu studied ^{19}F -NMR spectroscopy. *Eur. J. Biochem.* **218**: 1041–1047.
- Grage, S.L., Wang, J., Cross, T.A., and Ulrich, A.S. 2002. Solid-state ^{19}F -NMR analysis of ^{19}F -labeled tryptophan in gramicidin A in oriented membranes. *Biophys. J.* **83**: 3336–3350.
- Grzesiek, S. and Bax, A. 1992. Correlating backbone amide and side chain resonances in larger proteins by multiple relayed triple resonance NMR. *J. Am. Chem. Soc.* **114**: 6291–6293.
- Grzesiek, S., Anglister, J., and Bax, A. 1993. Correlation of backbone amide and aliphatic side-chain resonances in C-13/N-15-enriched proteins by isotropic mixing of C-13 magnetization. *J. Magn. Reson. Ser. B* **101**: 114–119.
- Guntert, P. 2004. Automated NMR structure calculation with CYANA. *Methods Mol. Biol.* **278**: 353–378.
- Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**: 51–59.
- Hull, W.E. and Sykes, B.D. 1974. Fluorotyrosine alkaline phosphatase. ^{19}F nuclear magnetic resonance relaxation times and molecular motion of the individual fluorotyrosines. *Biochemistry* **13**: 3431–3437.
- . 1975. Fluorotyrosine alkaline phosphatase: Internal mobility of individual tyrosines and the role of chemical shift anisotropy as a ^{19}F nuclear spin relaxation mechanism in proteins. *J. Mol. Biol.* **98**: 121–153.
- . 1976. Fluorine-19 nuclear magnetic resonance study of fluorotyrosine alkaline phosphatase: The influence of zinc on protein structure and a conformational change induced by phosphate binding. *Biochemistry* **15**: 1535–1546.
- Johnson, B.A. and Blevins, R.A. 1994. NMRView: A computer program for the visualization and analysis of NMR data. *J. Biomol. NMR* **4**: 603–614.
- Kover, K.E. and Batta, G. 1987. Theoretical and practical aspects of one-dimensional and two-dimensional heteronuclear Overhauser experiments and selective C-13 T1-determinations of heteronuclear distances. *Prog. Nucl. Magn. Reson. Spectrosc.* **19**: 223–266.
- Lau, E.Y. and Gerig, J.T. 1997. Effects of fluorine substitution on the structure and dynamics of complexes of dihydrofolate reductase (*Escherichia coli*). *Biophys. J.* **73**: 1579–1592.
- Li, M.X., Saude, E.J., Wang, X., Pearlstone, J.R., Smillie, L.B., and Sykes, B.D. 2002. Kinetic studies of calcium and cardiac troponin I peptide binding to human cardiac troponin C using NMR spectroscopy. *Eur. Biophys. J.* **31**: 245–256.
- Linge, J.P., Williams, M.A., Spronk, C.A., Bonvin, A.M., and Nilges, M. 2003. Refinement of protein structures in explicit solvent. *Proteins* **50**: 496–506.
- Lyons, B.A. and Montelione, G.T. 1993. An HCCNH triple-resonance experiment using C-13 isotropic mixing for correlating backbone amide and side-chain aliphatic resonances in isotopically enriched proteins. *J. Magn. Reson. Ser. B* **101**: 206–209.
- Pearlstone, J.R., Chandra, M., Sorenson, M.M., and Smillie, L.B. 2000. Biological function and site II Ca^{2+} -induced opening of the regulatory domain

- of skeletal troponin C are impaired by invariant site I or II Glu mutations. *J. Biol. Chem.* **275**: 35106–35115.
- Pratt, E.A. and Ho, C. 1975. Incorporation of fluorotryptophans into proteins of *Escherichia coli*. *Biochemistry* **14**: 3035–3040.
- Rinaldi, P.L. 1983. Heteronuclear 2D-NOE spectroscopy. *J. Am. Chem. Soc.* **105**: 5167–5168.
- Salopek-Sondi, B., Vaughan, M.D., Skeels, M.C., Honek, J.F., and Luck, L.A. 2003. ^{19}F NMR studies of the leucine-isoleucine-valine binding protein: Evidence that a closed conformation exists in solution. *J. Biomol. Struct. Dyn.* **21**: 235–246.
- Sattler, M., Schwendinger, M.G., Schleucher, J., and Griesinger, C. 1995. Novel strategies for sensitivity enhancement in heteronuclear multidimensional NMR experiments employing pulsed-field gradients. *J. Biomol. NMR* **6**: 11–22.
- Schwieters, C.D., Kuszewski, J.J., Tjandra, N., and Clore, G.M. 2003. The Xplor-NIH NMR molecular structure determination package. *J. Magn. Reson.* **160**: 65–73.
- Sia, S.K., Li, M.X., Spyropoulos, L., Gagné, S.M., Liu, W., Putkey, J.A., and Sykes, B.D. 1997. NMR structure of cardiac troponin C reveals an unexpected closed regulatory domain. *J. Biol. Chem.* **272**: 18216–18221.
- Sixl, F., King, R.W., Bracken, M., and Feeney, J. 1990. ^{19}F -NMR studies of ligand binding to 5-fluorotryptophan- and 3-fluorotyrosine-containing cyclic AMP receptor protein from *Escherichia coli*. *Biochem. J.* **266**: 545–552.
- Slupsky, C.M., Boyko, R.F., Booth, V.K., and Sykes, B.D. 2003. Smart-notebook: A semi-automated approach to protein sequential NMR resonance assignments. *J. Biomol. NMR* **27**: 313–321.
- Steinrücken, H.C. and Amrhein, N. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic-acid 3-phosphate synthase. *Biochem. Biophys. Res. Commun.* **94**: 1207–1212.
- Sykes, B.D. and Weiner, J.H. 1980. Biosynthesis and ^{19}F characterization of fluoroamino acid containing proteins. In *Magnetic resonance in biology* (ed. J.S. Cohen), pp. 171–196. John Wiley & Sons, New York.
- Trigo-Gonzalez, G., Racher, K., Burtnick, L., and Borgford, T. 1992. A comparative spectroscopic study of tryptophan probes engineered into high- and low-affinity domains of recombinant chicken troponin C. *Biochemistry* **31**: 7009–7015.
- Ulrich, A.S. 2005. Solid state F-19 NMR methods for studying biomembranes. *Prog. Nucl. Magn. Reson. Spectrosc.* **46**: 1–21.
- Valencia, F.F., Paulucci, A.A., Quaggio, R.B., Da Silva, A.C., Farah, C.S., and Reinach, F.C. 2003. Parallel measurement of Ca^{2+} binding and fluorescence emission upon Ca^{2+} titration of recombinant skeletal muscle troponin C. Measurement of sequential calcium binding to the regulatory sites. *J. Biol. Chem.* **278**: 11007–11014.
- Willard, L., Ranjan, A., Zhang, H., Monzavi, H., Boyko, R.F., Sykes, B.D., and Wishart, D.S. 2003. VADAR: A Web server for quantitative evaluation of protein structure quality. *Nucleic Acids Res.* **31**: 3316–3319.
- Wishart, D.S., Sykes, B.D., and Richards, F.M. 1991. Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. *J. Mol. Biol.* **222**: 311–333.
- Wittekind, M. and Mueller, L. 1993. HNCACB, a high-sensitivity 3D NMR experiment to correlate amide-proton and nitrogen resonances with the α - and β -carbon resonances in proteins. *J. Magn. Reson.* **B 101**: 201–205.
- Xiao, G., Parsons, J.F., Tesh, K., Armstrong, R.N., and Gilliland, G.L. 1998. Conformational changes in the crystal structure of rat glutathione transferase M1-1 with global substitution of 3-fluorotyrosine for tyrosine. *J. Mol. Biol.* **281**: 323–339.