The effect of the cosolvent trifluoroethanol on a tryptophan side chain orientation in the hydrophobic core of troponin C

Olivier Julien, Pascal Mercier, Melissa L. Crane, and Brian D. Sykes*

Department of Biochemistry, University of Alberta, Edmonton, AB, T6G 2H7, Canada

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Abstract: The unique biophysical properties of tryptophan residues have been exploited for decades to monitor protein structure and dynamics using a variety of spectroscopic techniques, such as fluorescence and nuclear magnetic resonance (NMR). We recently designed a tryptophan mutant in the regulatory N-domain of cardiac troponin C (F77W-cNTnC) to study the domain orientation of troponin C in muscle fibers using solid-state NMR. In our previous study, we determined the NMR structure of calcium-saturated mutant F77W-V82A-cNTnC in the presence of 19% 2,2,2-trifluoroethanol (TFE). TFE is a widely used cosolvent in the biophysical characterization of the solution structures of peptides and proteins. It is generally assumed that the structures are unchanged in the presence of cosolvents at relatively low concentrations, and this has been verified for TFE at the level of the overall secondary and tertiary structure for several calcium regulatory proteins. Here, we present the NMR solution structure of the calcium saturated F77W-cNTnC in presence of its biological binding partner troponin I peptide (cTnI_{144–163}) and in the absence of TFE. We have also characterized a panel of six F77W-cNTnC structures in the presence and absence TFE, cTnI_{144–163}, and the extra mutation V82A, and used ¹⁹F NMR to characterize the effect of TFE on the F77(5fW) analog. Our results show that although TFE did not perturb the overall protein structure, TFE did induce a change in the orientation of the indole ring of the buried tryptophan side chain from the anticipated position based upon homology with other proteins, highlighting the potential dangers of the use of cosolvents.

Keywords: NMR; rotamer; TFE; tryptophan; troponin C; side chain orientation

Abbreviations: 5fW, 5-fluorotryptophan; cCTnC, C-domain of cTnC; cNTnC, N-domain of cTnC; cTnC, human cardiac troponin C; cTnI_{144–163}, residues 144 to 163 of cTnI (cardiac switch peptide); HSQC, heteronuclear single quantum coherence; NMR, Nuclear Magnetic Resonance; NOE, nuclear Overhauser enhancement; RMSD, root mean square deviation; TFA, trifluoroacetate; TFE, 2,2,2-trifluoroethanol; TnC, troponin C; TnI, troponin I.

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*Correspondence to: Brian D. Sykes, 419 Medical Sciences Building, University of Alberta, Edmonton AB T6G 2H7, Canada. E-mail: brian.sykes@ualberta.ca

Introduction

The use of cosolvents in studies of biological macromolecules is a common practice in biochemistry and structural biology. Their use facilitates the study of small polypeptides as well as large molecular complexes of protein and nucleic acids. Cosolvents such as alcohols, glycerol, and others have been used in structure determination by X-ray crystallography and solution studies using circular dichroism and nuclear magnetic resonance (NMR) spectroscopy. In particular, 2,2,2-trifluoroethanol (TFE) is a widely used cosolvent in the determination of NMR solution structures of peptides and proteins. TFE is often used to induce secondary structure of small amino acid chains by

favoring the formation of α -helices or even β -sheet content.^{1,2} TFE has also been shown to stabilize β -hairpin structures³ and induce amyloid-like fibril formation.^{4,5} Furthermore, TFE is used as a membrane-mimetic solvent⁶ to solubilize and stabilize the structure of membrane peptides and proteins, and also to prevent their aggregation. TFE also decreases the tendency of proto-filaments to form clusters.⁷ Studies of the NMR solution structures of the calcium regulatory protein troponin C from skeletal and cardiac muscle^{8,9} have also demonstrated that TFE can be used as a denaturant of quaternary structure, weakening hydrophobic interactions within or between molecules in solution. In fact, the NMR solution structure of several troponin C constructs have been determined in the presence of TFE by ourselves and other groups,^{9–11} and more than 60 NMR structures in the Protein Data Bank (PDB) were determined in a mixture of water and TFE. For a complete review on the use of TFE and cosolvents, see Ref. 12. The extensive use of cosolvents such as TFE makes the understanding of their mechanism of action desirable, but the widely different examples suggest that the mechanism may be complex and unpredictable, which is the case in our present study.

The long-term goal of this research project is to obtain in situ structural information regarding the molecular changes of sarcomere proteins during muscle contraction. Many techniques and spectroscopic probes have been used to monitor the structure, function, and dynamics of the muscle protein machinery: for example, fluorescence resonance energy transfer measurements,¹³ electron paramagnetic resonance spectroscopy using nitroxide probes,¹⁴ electron microscopy,¹⁵ NMR spectroscopy,¹⁶ and X-ray crystallography.^{17,18} One recent and particularly powerful approach involves the use of bifunctional rhodamine fluorescence labels to monitor protein domain orientation in situ. Examples include the orientation of the domains of myosin light chains and troponin C (TnC) in active muscle fibers.^{19,20} However, these bifunctional rhodamine probes are bulky and hydrophobic with a proclivity for complex dynamics.^{21,22} The use of solid-state NMR methods is rapidly becoming important in many immobilized biological systems such as membrane proteins in phospholipid bilayers. In NMR, the probe is a nuclear spin (either naturally occurring or isotopically labeled). Even when a non-natural nuclear spin label such as ¹⁹F is used, it is expected to be much less perturbing than the larger aromatic optical or electron spin resonance probes.

For more than half a century, tryptophan fluorescence has been a popular method to study protein–protein interactions, protein–drug bindings or to monitor other conformational changes affecting protein structures (see Ref. 23 for a review on protein fluorescence). For example, tryptophan fluorescence has been extensively used to study the effect of calcium binding to chicken skeletal troponin C with the engineering of sev-

eral tryptophan mutants such as F29W,²⁴ and F78W and F154W.^{25,26} Similar studies also exploited the mutation F102W in parvalbumin and oncomodulin.^{27,28} Over the past few years, we have designed several tryptophan mutants of cardiac troponin C to incorporate ¹⁹F-labeled tryptophan and then to use solid-state ¹⁹F NMR spectroscopy to determine the orientation and dynamics of TnC during calcium triggered muscle contraction.^{10,29,30} The design criterion was to incorporate a single tryptophan immobilized within the core of protein in a presumed well-defined position. TnC is a calcium-binding protein containing several EF hand motifs, which are formed by a contiguous stretch of 12 residues with six of them involved in calcium binding, denoted X, Y, Z, –Y, –X, and –Z at positions 1, 3, 5, 7, 9, and 12. The immediately preceding and following residues are always hydrophobic, with the subsequent residue virtually always being a phenylalanine. The EF hands are virtually always two site domains, even if sometimes the individual sites no longer bind metals. The phenylalanine residues following an EF hand are buried in middle of the domain and serve to anchor the Ca²⁺ binding site. In some cases, the phenylalanine residue is replaced by a naturally occurring tryptophan in this location, as found in silver hake Parvalbumin and scallop TnC.^{31,32} We have previously determined the structures of F153W and F153(5fW) cardiac troponin C,²⁹ and demonstrated that the indole side chain in this position is immobilized and in an expected orientation compared with silver hake Parvalbumin W102. Recently, we described the effect of the F77W substitution on the 3D structure, troponin I affinity, and in situ activity of troponin C.¹⁰ Interestingly, the NMR structure of the F77W-V82A mutant of the N-domain of troponin C in the presence of 19% TFE revealed that the W77 side chain was in a different orientation compared with the one found in F153W-cTnC and silver hake parvalbumin, suggesting a rationalization for the altered biological activity. The presence of TFE was used there to counteract the higher tendency of the mutant to dimerize compared with the wild type protein. The importance of tryptophan side chain orientations in biology has been recently highlighted by two studies. Ozkirimli *et al.*³³ have shown using enzyme kinetics and molecular dynamics simulations that the activation of Src kinase is coupled with the side chain conformer of W260. Using ¹⁹F solid-state NMR spectroscopy, Witter *et al.*³⁴ have shown that the activation/inactivation of protein M2 of influenza A virus is regulated by the side chain rotamer of the W41.

We have determined the NMR solution structure of the calcium saturated mutant F77W-cTnC in the absence of TFE and in complex with its binding partner, residues 144 to 163 of troponin I (cTnI_{144–163}). We have structurally characterized a panel of structures of F77W-cTnC in the presence and absence of TFE, cTnI_{144–163}, and V82A. Interestingly, the tryptophan orientation of W77 in the F77W-cTnC:cTnI_{144–163}

Figure 1. Opposite side chain orientation of W77 in water and TFE (A) Solution structure of calcium saturated mutant F77W-V82A of N-domain cardiac troponin C (cNTnC) determined in 19% TFE (v/v).¹⁰ The side chain of W77 and residues at close proximity are shown in ball-and-sticks. The carbon atoms are colored in green, the hydrogens in white, the nitrogens in blue, and the calcium ion in black. The W77 side chain orientation revealed an indole ring pointing towards helices N and A ($\pm 90^\circ$). (B) Solution structure of calcium saturated F77W-cNTnC determined in H₂O and bound to its protein binding partner cTnI_{144–163} (shown in red). The W77 side chain orientation revealed an indole ring pointing towards helices B and C ($\pm 105^\circ$). In comparison to A), the W77 indole ring is rotated close to 180° around its C β -C γ bond. PyMOL (DeLano Scientific LLC) was used to make these figures.

complex was observed to be in the canonical orientation observed for F153W-cCTnC and W102 in silver hake parvalbumin, and opposite to the orientation determined for F77W-V82A-cNTnC in the presence of 19% TFE. Of the three possibilities that could cause this conformational difference, the panel of structures presented herein revealed that the TFE is responsible for the different orientation of the indole side chain of F77W. We have also used ¹⁹F NMR to characterize the effect of TFE on the F77(5fW) analog.

Results

The focus of this article is to evaluate the effect of TFE on the structure of cNTnC by comparing NMR data obtained for the mutant F77W in the presence and absence of the mutation V82A, the cosolvent TFE, and the binding partner, residues 144 to 163 of troponin I (cTnI_{144–163})*. The previously determined NMR solu-

tion structure of F77W-V82A-cNTnC in 19% TFE is shown in Figure 1(A). In this structure, the orientation of the indole ring is inconsistent with that observed in the calcium saturated F153W-cCTnC²⁹ and silver hake parvalbumin W102³¹ [Fig. 1(A) insert]. In this article, we describe the new NMR solution structure of the F77W-cNTnC-cTnI_{144–163} complex in the absence of TFE [Fig. 1(B)]. In this new structure, the tryptophan indole ring is in the canonical orientation with a NH indole pointing toward helices N and A [Fig. 1(B) inset], as observed for F153W-cCTnC and parvalbumin. To further understand the effect of the trifluoroethanol on the structure of cardiac troponin C, we have structurally characterized a panel of structures in the presence and absence of TFE, V82A, and cTnI_{144–163} peptide; and we have used ¹⁹F NMR to characterize the effect of TFE on the F77(5fW) analog.

The overall fold of the newly determined NMR solution structure of F77W-cNTnC-cTnI_{144–163} in the absence of TFE is very similar to the previously determined structure of F77W-V82A-cNTnC in 19% TFE

*All of the calcium-binding proteins described herein were all studied in their calcium-saturated states.

Table I. Structural Statistics for F77W-cNTnC in the Calcium Saturated F77W-cNTnC-cTnI₍₁₄₄₋₁₆₃₎ Complex for the 20 Best Target-Function Structures

Intramolecular NOE restraints	
Total	1280
Short range ($ i - j \leq 1$)	747
Medium range ($1 \leq i - j < 5$)	342
Long range ($ i - j \geq 5$)	191
Dihedral restraints	
Total	134
ϕ	67
ψ	67
χ_1	0
Restraint violations	
Distance $> 0.2 \text{ \AA}$	4
Dihedral $> 1^\circ$	0
RMSD to averaged coordinates ($\text{\AA}$)	
Well defined regions (N, C $_{\alpha}$, C) ^a	0.77
All regions (N, C $_{\alpha}$, C)	0.95
N helix (5–11)	0.14
A helix (14–27)	0.36
B helix (41–48)	0.14
C helix (54–62)	0.23
D helix (74–85)	0.30
Average cyana target function	0.55
ϕ , ψ in core or allowed regions ^a	
Residues in most favoured regions	91.8%
Residues in additional allowed regions	8.2%
Residues in generously allowed regions	0.0%
Residues in disallowed regions	0.0%

^a Using residues 5–85.

(2jxl) and that of the wild type calcium saturated cNTnC-cTnI₁₄₇₋₁₆₃ complex (1mxl). The structural statistics for the structure are presented in Table I, and the ensemble of NMR structures deposited to the PDB with access code [2kgb.pdb](#). The RMSD for the superimposition of the backbone atoms for residues 5–85 of F77W-cNTnC-cTnI₁₄₄₋₁₆₃ with F77W-V82A-cNTnC in 19% TFE and cNTnC-cTnI₁₄₇₋₁₆₃ is 1.6 and 2.2 $\text{\AA}$, respectively. These relatively low-RMSD values indicate that the backbones of the three proteins are very similar to each other. Of particular interest is the orientation of the side chain of residue 77. Amino acid side chains are usually described using two dihedral angles χ_1 and χ_2 , defined by the N-C $_{\alpha}$ -C $_{\beta}$ -C $_{\gamma}$ and C $_{\alpha}$ -C $_{\beta}$ -C $_{\gamma}$ -C $_{\delta 1}$ bonds, respectively. The tryptophan in F77W-cNTnC-cTnI₁₄₄₋₁₆₃ has χ_1 and χ_2 values of -171° and -110° , respectively. In general, residues with two H β preferentially adopt rotamers that have one of the three standard χ_1 values of $-60^\circ \pm 30^\circ$, $60^\circ \pm 30^\circ$, and $180^\circ \pm 30^\circ$, often referred to in the literature as gauche negative (g $-$ or m), gauche positive (g $+$ or p) and trans (t), respectively.³⁵⁻³⁷ For a tryptophan like W77, the combination of the three standard χ_1 and two possible χ_2 values leads to six favorable rotamers.^{38,39} Accordingly, all of the tryptophan side chains discussed herein for troponin C (with and without TFE) and parvalbumin (W102) possess a trans orientation with similar χ_1 dihedral angles. This leads to virtually coplanar aromatic rings in all structures. However, the orientation of W77 in the structure in

19% TFE is in the opposite direction with respect to W77 in F77W-cNTnC-cTnI₁₄₄₋₁₆₃ and W153 in F153W-cCTnC (both in the absence of TFE). This difference can be explained by their opposite χ_2 values. Using the nomenclature described by Lovell *et al.*, the tryptophan orientation of F77W-V82A-cNTnC in 19% TFE can be categorized as t90 $^\circ$ (χ_1 = trans and $\chi_2 \sim 90^\circ$) in comparison to t-105 $^\circ$ (χ_1 = trans and $\chi_2 \sim -105^\circ$) for F77W-cNTnC-cTnI₁₄₄₋₁₆₃, F153W-cCTnC and silver hake parvalbumin W102.

To determine the cause(s) of the reorientation of this buried tryptophan side chain, we collected structural data for the panel of F77W samples in the presence and absence of cTnI₁₄₄₋₁₆₃, V82A, or TFE. We acquired 3D ^{13}C NOESY-HSQC NMR spectra for all of the samples to look at the differential NOE contacts of the W77 indole ring. Figure 2 shows the NOE contacts between the W77.HD1 (C2') and its surrounding protons for different sample conditions. By comparing Figure 2(A,E), one can clearly see the different NOE patterns for F77W-V82A-cNTnC in 19% TFE (t90 $^\circ$) and F77W-cNTnC-cTnI₁₄₄₋₁₆₃ (t-105 $^\circ$). A complete set of the unambiguous NOEs observable between W77 in a t90 $^\circ$ and a t-105 $^\circ$ orientation is presented in Table II. Using these NOE patterns for the different aromatic protons of the tryptophan, one can clearly identify the indole ring orientation of residue 77 in a specific sample condition or within a given protein complex. Figure 2(D) clearly shows that the indole orientation of F77W-cNTnC in water and in the absence of cTnI results in NOE contacts similar to Figure 2(E), where cTnI₁₄₄₋₁₆₃ is present. This is typical of an indole t-105 $^\circ$ situation, meaning that the presence of cTnI₁₄₄₋₁₆₃ is not the key for the W77 orientation change. Focusing on the V82A mutation, we looked at the aromatic NOE contacts of F77W-V82A-cNTnC in water without TFE or peptide [Fig. 2(C)]. Again, the NOE contacts were typical of a W77 indole having a χ_2 of -105° , ruling out the V82A mutation as being responsible for the aromatic flip. To confirm that the presence of TFE is the cause of the W77 orientation change, we compared the NOE contacts of both F77W and F77W-V82A in 19% TFE [Fig. 2(A,B)] and observed similar NOE contacts, both characteristic of a W77 in a t90 $^\circ$ rotamer conformation. These results demonstrated that TFE is responsible for the tryptophan indole orientation change.

To investigate the effect of the concentration of TFE on the W77 aromatic ring orientation, we titrated the protein with TFE from 0 to 25% (v/v) and monitored the structural changes on F77W-cNTnC. For each step in the titration, a 2D ^{15}N HSQC NMR spectrum and a 2D ^{13}C HSQC NMR spectrum of the aromatic region were acquired to look at the effect of TFE on the chemical environment of W77 [Fig. 3(A)]. The results suggest that early in the titration (i.e., $\sim 2\%$ TFE) a conformational change occurs; as evidenced by the absence of some of the aromatic proton signals

Figure 2. W77 CD1 planes of 3D ^{13}C NOESYHSQCs acquired at 600 MHz. The observable NOEs in the presence of TFE are shown in panels A and B, for F77W-V82A-cNTnC and F77W-cNTnC, respectively, revealing a W77 rotamer in the t90° position ($\chi_1 = \text{trans}$ and $\chi_2 \sim 90^\circ$). In opposition, the spectra acquired in H_2O are presented in panels C, D and E, for F77W-V82A-cNTnC, F77W-cNTnC and F77W-cNTnC-cTnI_{144–163}, respectively. These are characteristic of an tryptophan indole occupying a t–105° rotamer position ($\chi_1 = \text{trans}$ and $\chi_2 \sim -105^\circ$).

like W77.HZ3 [Fig. 2(C)] and W77.HE1 (from ^{15}N HSQC data not shown). Both protons do not reappear until late in the titration around 19% TFE. Similarly, the W77.HZ2 signal broadens from 0 to about 10%, and starts to sharpen again as the titration continues. Other aromatic positions show different patterns, but most seem to present at two-step transition. For example, the proton W77.HD1 shows similar broadening to HZ2, but the chemical shift changes direction at 10% TFE and reverts toward its original position (that of 0% TFE) at the end of the titration.

We also looked at the effect of TFE on the methyl group of the methionine side chains by following the 2D ^{13}C HSQC NMR spectra of the aliphatic region during the TFE titration [Fig. 3(E)]. The spectral changes reveal that the side chain methyl resonances of M80 and M81 are by far the most perturbed by the increased concentration from 0 to 25% TFE. The total magnitude of the ^1H and ^{13}C chemical shift changes ($\Delta\delta_{\text{total}} = \sqrt{((\Delta\delta_{^1\text{H}})^2 + (\Delta\delta_{^{13}\text{C}})^2)}$) of M80 and M81 are 149 and 448 Hz, respectively, on a 500 MHz NMR spectrometer. These two residues are located in the hydrophobic pocket of cNTnC in close proximity to the indole ring of W77. Interestingly, the resonance for the methyl group of M81 is too broad to be observed between 2 and 19% TFE, as was observed for W77.HZ3.

To support these results, we have substituted the proton at position 5' of W77 (W77.HZ3) with a ^{19}F atom by the incorporation of 5-fluorotryptophan (5fW) at position 77 in order to monitor the effect of TFE on the tryptophan using ^{19}F NMR. The 1D ^{19}F NMR spectra obtained during the TFE titration of F77(5fW)-cNTnC are presented on Figure 4. At 0% TFE, the 5fW resonance has a chemical shift of -47.14 ppm. The peak is very broad with a linewidth ($\Delta\nu$) of 465 Hz. As the TFE concentration is increased, the ^{19}F resonance moves upfield and gets sharper, ending up at -47.87 ppm in 25% TFE with a $\Delta\nu$ of 78 Hz. This is roughly the linewidth expected for a protein the size of

Table II. Comparison of the W77 Aromatic Protons Inter-Residual NOEs Observed in TFE and H_2O

NOEs	t90° rotamer (TFE)	t–105° rotamer (H_2O)
W77.HD1	I36.MD, V72.QG, M80.ME	L78.HG, L78.QD
W77.HZ2	A23.MB, M81.ME	A23.MB, L41.QD
W77.HH2	A23.MB	L41.QD
W77.HZ3	F20.MB	
W77.HE3	F20.QD, F24.QD, W77.HA, L78.HN, L78.HA, L78.QB, L78.QD, M80.ME, M81.ME	I36.MD, V72.QG

Figure 3. Titration of F77W-cNTnC with the cosolvent TFE monitored on a 500 MHz NMR spectrometer. A) and E) show the superimposition of the ^{13}C -HSQCs for the aromatic region and methionine methyl regions, respectively. From the light to dark blue correspond to 0–25% TFE (v/v). B–D) 1D trace of W77.HZ2, W77.HZ3, and W77.HD1 cross-peaks, respectively. The peak intensities were adjusted to take into account the dilution factors caused by the addition of TFE.

F77(*5fW*)-cNTnC. When the cTnI_{144–163} peptide is bound to the protein (without TFE), the *5fW* resonance is also sharper, with a linewidth of 138 Hz. These results are consistent with exchange broadening of the ^{19}F NMR linewidth of the *5fW* residue in the absence of either TFE or cTnI_{144–163}. This broadening is caused by equilibrium between the t_{90° and t_{-105° conformations such that both TFE and cTnI_{144–163} force the equilibrium to one side or the other.

Discussion

More than 60 NMR structures in the PDB were determined in a mixture of water and TFE. The implicit assumption in these structures is that the structures are not changed by this cosolvent. For example, in our previous description of the effect of the F77W substitution on the 3D structure, troponin I affinity, and in

situ activity of troponin C,¹⁰ we modeled the TnC-TnI complex without questioning the unusual orientation of the tryptophan side chain observed in TFE. In this manuscript, we have demonstrated that TFE can change the orientation of a presumably buried and immobile tryptophan side chain by comparing the NMR solution structures of F77W-cNTnC·cTnI_{144–163} and F77W-cNTnC in the presence of 19% TFE. Using NOESY experiments, we ruled out the effect of the V82A mutation and the presence of the bound peptide in the hydrophobic pocket, and identified the cosolvent TFE to be responsible for the change in the indole ring orientation.

How does TFE change the orientation of W77? One possibility is that the hydrophobic TFE molecules bind to and disturb the hydrophobic pocket of cNTnC. This could involve a general nonspecific binding or a binding to a specific site(s). In the former case, the

Figure 4. ^{19}F NMR spectra of F77(5fW)-cNTnC acquired during the titration with the cosolvent TFE (from 0 to 25% TFE). The spectra were acquired on a 600 MHz spectrometer with a $^1\text{H}/^{19}\text{F}$ probe. The spectrum at the top does not contain TFE, but is saturated with cTnI_{144–163}. The peak intensities were adjusted to take into account the dilution factors caused by the addition of TFE. Chemical shifts are with respect to TFA at 0 ppm. The resonance near -46 ppm arises from the AEBF included in the cocktail of protease inhibitors.

binding of TFE could lead to a change in the equilibrium between the open and closed states of cNTnC existing in solution.¹⁶ Inspection of the structure of F77W-V82A-cNTnC in 19% TFE reveals that the N-domain has a slightly more open structure compared with wild type, which might allow more space for the W77 and facilitate the rotation of aromatic side chain. In the absence of TFE, the tryptophan indole NH is solvent-exposed and forms hydrogen bonds, or at least favorable electrostatic interactions, with the water molecules, whilst the bulky hydrophobic ring is buried inside the protein core making favorable contact with I36 and V72 [Fig. 1(B)]. However, when TFE is present, the hydrophobic aromatic ring of W77 could make favorable interactions with the TFE molecules.

In the case of a specific binding site, TFE may bind to the methionine side chains as previously suggested for a similar molecule, 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane).⁴⁰ This study suggested that the anesthetic molecule was bound between the side chains of a methionine and a tryptophan; the

$-\text{CF}_3$ of the drug making favorable contacts with the sulfur atom of the methionine side chain in the core of a four α -helix bundle. In our case, there is a surface pocket in the structure formed by M80, M81, and W77. The large chemical shift changes observed for the methyl groups of M80 and M81 support this possibility. However, the changes in chemical shifts for these two residues could also be a consequence of a change in the orientation of an aromatic ring, which would occur with a W77 indole flip in close proximity. We have, however, been unable to observe any clear heteronuclear ^1H - ^{19}F NOE contacts between the CF_3 group of TFE and either the M80 and M81 methyl groups or the W77 ring protons (data not shown).

Previous studies using tryptophan fluorescence measurements and molecular dynamics simulations on the F78W mutant of skeletal TnC reported the possibility of two or more side chain orientations for the homologous tryptophan (skeletal F78W mutation is equivalent to cardiac F77W).^{25,26} The minimum perturbation map of W78 χ_1 and χ_2 presented by Moncrieffe *et al.* suggests two minimum wells of $\chi_1 \times \chi_2$ corresponding to the two tryptophan indole orientations described in this article. Figure 5 shows the $\chi_1 \times \chi_2$ isomerization map of the different tryptophans naturally occurring and engineered into troponin C and parvalbumin. Of course, the conformations of all protein side chains are dynamic in solution, so that equilibrium between different indole conformations for W77 presumably exists in solution. In water, the main conformation of the W77 indole ring is oriented towards helices B and C as observed in the F77W-cNTnC-cTnI_{144–163} structure. With the addition of a

Figure 5. Tryptophan side chain $\chi_1 \times \chi_2$ conformations in A) F77W-cNTnC-cTnI_{144–163} B) F77W-V82A-cNTnC in 19% TFE C) F153W-cTnC and D) silver hake parvalbumin W102. The dark regions correspond to the most favorable conformations. The figure was adapted from the figure obtained using procheck nmr.⁴¹

small amount of TFE (i.e., 2% TFE), the equilibrium of the aromatic moiety is perturbed as reflected by the exchange broadening of some aromatic proton resonances in the ^{13}C and ^{15}N HSQC NMR spectra. By adding more TFE (i.e., $\sim 19\%$ TFE), the equilibrium is then shifted toward the opposite indole orientation.

Obviously, if TFE changes the orientation and dynamics of a buried tryptophan side chain, it will most likely affect a number of other residues. However, this usually does not have a major impact on the overall structure of the protein, since the side chains that are solvent accessible are most often flexible and poorly defined in solution. The caution is to be very careful when making mechanistic conclusions based upon the orientation of surface residues. Our results reemphasize that proteins are highly dynamic, and support a multitude of previous fluorescence studies that indicated that tryptophan rings are not restrained to only one conformation even if well packed in a protein.

Materials and Methods

Protein expression and purification

pET3A plasmids containing the sequence for F77W-cNTnC (1–89) or F77W, V82A-cNTnC (1–89) were transformed into BL21(DE3)pLysS host cells. The expression and purification of [$U\text{-}^{13}\text{C}$, ^{15}N]-labeled protein in *E. coli* has previously been described.^{42,43} Decalcification was performed as described.⁴³ Expression of F77(5fW)-cNTnC was performed as described²⁹ and purified as described.⁴³ The human cardiac troponin I (cTnI) peptide containing residues 144 to 163 (Ac-RRVRISADAMMQALLGARAK-NH₂) was bought from GL Biochem (Shanghai, China).

NMR sample preparation

The contents of the different NMR samples used in this study are described below. A cocktail of protease inhibitors (Calbiochem) and NaN₃ were used to prevent sample degradation. The NMR buffer consists of 100 mM KCl and 10 mM imidazole in H₂O. Dithiothreitol (DTT) was used to prevent the oxidation of cysteines and CaCl₂ to saturate calcium binding site I. The pH of the sample was calculated from the measurement of the chemical shift of the imidazole H2 proton.⁴⁴ Every ^1H spectrum was referenced according to the DSS-*d*₆ signal (Chenomx, Canada). Standard 5 mm NMR tubes with 500 μL of solution were used unless specified otherwise.

The following samples were prepared to obtain NMR data of the different proteins: (1) F77W-V82A-cNTnC: 4.2 mg [$U\text{-}^{13}\text{C}$, ^{15}N]-labeled protein, 500 μL NMR buffer, 0.3 mM DSS-*d*₆, 0.03% NaN₃, 6 mM CaCl₂, 15 mM DTT, 6.0 μL protease inhibitors. The pH was adjusted to 6.40. (2) F77W-cNTnC: 4.0 mg [$U\text{-}^{13}\text{C}$, ^{15}N]-labeled protein, 500 μL NMR buffer, 0.3 mM DSS-*d*₆, 0.015% NaN₃, 6 mM CaCl₂, 15 mM DTT,

6.0 μL protease inhibitors. The pH was adjusted to 6.76. (3) F77W-V82A-cNTnC-TFE: 5.3 mg [$U\text{-}^{13}\text{C}$, ^{15}N]-labeled protein, 215 μL NMR buffer, 19% TFE, 0.025% NaN₃, 0.2 mM DSS-*d*₆, 6 mM CaCl₂, 10 mM DTT, 3.0 μL protease inhibitors. The pH was adjusted to 6.80 and the sample transferred into a Shigemi tube (Shigemi Ltd., Japan). (4) F77W-cNTnC-TFE: 5.2 mg [$U\text{-}^{13}\text{C}$, ^{15}N]-labeled protein, 245 μL NMR buffer, 60 μL TFE, 0.3 mM DSS-*d*₆, 0.025% NaN₃, 6 mM CaCl₂, 16 mM DTT, 3.0 μL protease inhibitors. The pH was adjusted to 6.71 and the sample transferred to a Shigemi tube (Shigemi, Japan). (5) F77W-cNTnC-cTnI_{144–163}: 3.7 mg [$U\text{-}^{13}\text{C}$, ^{15}N]-labeled protein, 500 μL NMR buffer, 0.3 mM DSS-*d*₆, 0.03% NaN₃, 6 mM CaCl₂, 15 mM DTT, 6.0 μL protease inhibitors, and 1.7 mg cTnI_{144–163}. The pH was adjusted to 6.79. (6) TFE Titration of F77W-cNTnC: 6.1 mg [$U\text{-}^{13}\text{C}$, ^{15}N]-labeled protein, 500 μL NMR buffer, 0.3 mM DSS-*d*₆, 6 mM CaCl₂, 15 mM DTT, 0.03% NaN₃, and 6 μL protease inhibitors. The pH was adjusted to 6.69. An identical sample was prepared to titrate F77(5fW)-cNTnC with TFE, and another one to examine the effect of cTnI_{144–163}.

NMR data acquisition and processing

The ^1H , ^{13}C , ^{15}N NMR spectra presented in this study were acquired on Varian INOVA 500, 600, and 800 MHz NMR spectrometers (the latter equipped with a cryogenic probe) using Biopack pulse sequences (version 2008-01-18, VnmrJ 2.1B, Varian). Each spectrum presented in Figure 2 was obtained with the gnoesyChsqc pulse sequence⁴⁵ and acquired with 16 scans at a resolution of $512 (t_3) \times 64 (t_2) \times 32 (t_1)$ or more complex points. The spectral widths were 14 or 10 ppm for the ^1H -dimensions, and 20 or 30 ppm for the ^{13}C -dimension with a carrier position at 125 ppm specific for the aromatic region. For the TFE titration (Fig. 3), a 2D ^1H - ^{13}C HSQC with 32 scans was acquired with a resolution of $512 (t_2) \times 128 (t_1)$ complex points for every of step of the titration: 0, 2, 4, 6, 8, 10, 14, 19, and 25% TFE (v/v). All of the NMR spectra acquired in this study were processed with NMRPipe⁴⁶ or vnmrJ 2.1B (Varian). Prior to the Fourier transform, a sinebell function of 60 to 90° offsets was applied, and then zero filling was used to extend a maximum of twice the number of real points. Linear prediction was only used for the t_3 dimension of 3D spectra with a maximum of half the number of acquired points predicted. The chemical shift assignments were completed using with smartnotebook v5.1.3⁴⁷ and NMRViewJ (One Moon Scientific).

The ^{19}F NMR spectroscopy was performed on the Varian INOVA 600MHz, by replacing the gradient probe with our home-built $^{19}\text{F}/^1\text{H}$ probe.⁴⁸ Each ^{19}F NMR spectrum presented in Figure 4 was acquired with 1000 scans, a spectral width of 50,000 Hz, and a time delay of 1 s. The frequency of the trifluoroacetate (TFA) was used as an internal standard to reference

the spectra at 0 ppm. The spectra were processed with VnmrJ 2.1B (Varian inc.) using a line broadening of 40 Hz and back linear prediction for six points.

NMR structure determination of F77W-cNTnC-cTnI_{144–163}

The following spectra were used for the chemical shift assignments: 2D ¹H-¹⁵N HSQC, 2D ¹H-¹³C HSQC, 3D ¹H-¹⁵N TOCSY-HSQC, 3D CBCACONNH, 3D HNCACB for the backbone and 3D CCONH, 3D HCCONH, 3D HCCH-TOCSY for the side chains. The intramolecular NOE restraints for F77W-cNTnC (1284 total) were obtained from a 3D ¹⁵N NOESY-HSQC and two 3D ¹³C NOESY-HSQC (aliphatic and aromatic) acquired at 800 MHz. The 39 intramolecular NOE distances for cTnI_{144–163} were taken from a previously determined NMR structure involving cNTnC and cTnI_{147–163} (PDB access code 1lxf), as well as the 30 intermolecular distances used to position the peptide on the protein. The structure calculations were performed using CYANA 2.1.⁴⁹ The NOEs were calibrated within CYANA. The minimum and maximum NOE calibration values were set to 1.8 and 6.0 Å, respectively. In addition, eight calcium-binding restraints (based on calcium binding site homology) and 134 dihedral restraints from TALOS⁵⁰ were used to obtain the NMR ensemble. The option autoaco was also used in cyana to generate additional angle constraints. The structure validation was performed using procheck v.3.5.4.⁴¹ The favorable dihedral angles, restraint violations, and cyana target function are presented in Table I.

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