Placement of ^{19}F into the center of GB1: effects on structure and stability

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Abstract A structural and thermodynamic characterization of 5F-Trp-substituted immunoglobulin binding domain B1 of streptococcal protein G (GB1) was carried out by nuclear magnetic resonance and circular dichroism spectroscopy. A single fluorine reporter atom was positioned at the center of the three-dimensional structure, uniquely poised to be exploited for studying interior properties of this protein. We demonstrate that the introduction of 5F-Trp does not affect the global and local architecture of GB1 and has no influence on the thermodynamic stability. The favorable properties of the fluorinated GB1 render this molecule a desirable model system for the development of spectroscopic methodology and theoretical calculations.

Key words: Immunoglobulin G binding domain B1 of streptococcal protein G; Fluorine substitution; Protein structure; Nuclear magnetic resonance

1. Introduction

Over the last decade, several model systems have emerged which have found widespread use for studying a large variety of intrinsic protein properties. One such protein is the immunoglobulin binding domain B1 of streptococcal protein G (GB1) [1]. GB1 is a small (56 residues), stable, single domain protein with one α-helix and a four-stranded β-sheet comprised of two hairpins (see Fig. 1). A single tryptophan residue (Trp43) is located on β-strand 3, and the local environment of W43 in the native state is mostly hydrophobic. Its side chain is in van der Waals contact (< 5 Å) with two aromatic and three aliphatic residues. Thus, W43 is involved in the formation of the interface between the α-helix through contacts with F30 and A34 and the neighboring β-strands through interactions with L5, F52, V54. Since W43 occupies a critical position in the hydrophobic core of GB1, its properties and behavior have been exploited for investigating protein folding using tryptophan fluorescence spectroscopy [2].

The advantage of using the fluorine nucleus as a nuclear magnetic resonance (NMR) probe for studying proteins is well known [3,4]. Its relatively small size (van der Waals radius = 1.47 Å; only 0.15 Å larger than hydrogen), a nuclear spin of 1/2, 100% natural abundance and high NMR sensitivity (83% of that of a proton) make it a very attractive nucleus for NMR studies of many complex molecules. Because of the ability of the ^{19}F lone pair electrons to participate in non-bonded interactions with the local environment, ^{19}F chemical shifts are sensitive to changes in van der Waals contacts, electrostatic fields and hydrogen bonding present in biological macromolecules, particularly proteins.

Despite these attractions, ^{19}F NMR has mainly occupied a niche position in the very successful application of NMR to biomolecular systems. One of the reasons for past concerns pertains to perturbations caused by the fluorine atom itself, given its hydrogen bond acceptor properties (contrasting a simple hydrogen), and associated changes in dipole moment or pK_a for fluorine-substituted amino acids. Indeed, conflicting reports as to the effects of fluorination on structure and stability of proteins can be found in the literature [5,6]. More recently, reservations with respect to structural effects were shown to be unfounded, at least for F-Trp-substituted annexin V [7] and 5F-Trp-substituted TIM [8], whose X-ray structures were indistinguishable from those of wild-type protein. For annexin V, however, changes in thermodynamic stability and activity were observed, despite no observable structural effects [7]. Here we investigate the incorporation of 5F-Trp into wild-type GB1 and a GB1 mutant. The mutant (Y3F,Y45F) is part of a set of proteins designed for energy-transfer measurements between the single tryptophan and single, modified tyrosine residues in GB1. We demonstrate that no discernible effects on the structure or the thermal stability are caused by fluorination of both proteins.

2. Materials and methods

2.1. Chemicals and growth medium:

5-F-DL-Trp and all other amino acids were purchased from Sigma-Aldrich. The defined medium for F-Trp labeling contained each amino acid at concentrations ranging from 0.1 to 2.1 mg/l and 50 mg/l

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Abbreviations: GB1, immunoglobulin G binding domain B1 of streptococcal protein G; CD, circular dichroism; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence; HOESY, heteronuclear Overhauser and exchange spectroscopy

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5-F-DL-Trp. The amount of 5-F-DL-Trp in the growth medium was increased to 500 mg/l after induction.

2.2. Mutagenesis and protein purification

Replacement of tyrosines by phenylalanines was carried out using the QuikChange™ Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Initially, single substitutions at each of the tyrosine sites (Y3 and Y33) were introduced into the GB1 wild-type gene, residing on the pET11a vector. These single-site mutants were sequenced and the clones were tested for expression. The single-site mutant clones were used as templates for a second round of site-directed mutagenesis. Nucleotide sequences of the final cloned DNAs were confirmed by sequencing. All proteins were expressed in *Escherichia coli* BL-21 (DE3) using the pET11a vector system (Novagen Inc., WI, USA).

Cultures were grown at 37°C in a modified defined medium for uniform (>90%) ^{19}F labeling using excess 5-FTrp as the sole source for tryptophan. Cells were harvested 3–4 h after induction and frozen at −80°C. Frozen cells were thawed by suspending in PBS buffer (1 g cells/5 ml buffer) at 0°C. The cell suspension was heat-treated at 80°C for 5 min and immediately chilled on ice for 10 min. Precipitated denatured proteins as well as cell debris was removed by centrifugation in an SS34 rotor at 14 000 rpm. The supernatant contained predominantly GB1, which was purified further by reversed-phase high-performance liquid chromatography on POROS 20 R2 resin (PerSeptive Biosystems, Framingham, MA, USA) using a linear water/acetonitrile gradient. The identity and final purity of each mutant protein was verified by mass spectrometry and SDS–PAGE. The percentage labeling was established by NMR (see Fig. 2) and mass spectrometry. The F-substituted wild-type GB1 exhibited a molecular mass of 6240.5 g/mol, closely matching the expected mass of 6240.9 g/mol. Since the accuracy of he mass determination is ±1 mass unit, this corresponds to ca. 95% F-labeling in agreement with the percentage labeling determined by NMR. For storage, all proteins were dialyzed into water or 50 mM sodium phosphate buffer, pH 5.5, and kept at 4°C.

2.3. Circular dichroism (CD) spectroscopy

Purified protein was dissolved in 50 mM sodium acetate buffer, pH 5.5. The protein concentration was estimated by absorbance at 280 nm using a Hewlett-Packard 8452A diode array spectrophotometer using an extinction coefficient of 1.32/mg/ml. CD measurements were carried out on a J-720 spectropolarimeter (Jasco, Japan) equipped with a temperature control system (Neslab, USA). Far-UV spectra were recorded over 200–260 nm in a 0.1 cm cell for ~0.1–0.5 mg/ml protein samples. Thermal unfolding was followed at 222 nm using a heating/cooling rate of 1°C/min with a temperature step of 0.5°C.

2.4. NMR spectroscopy

All spectra were recorded on Bruker DMX500 and DMX600 spectrometers equipped with three-axes pulse field gradients and auto-shielded triple resonance probes at 25°C, except for the ^{1}H–^{19}F heteronuclear Overhauser and exchange spectroscopy (HOESY) spectra which were recorded at 22°C on a Varian Inova 500 spectrometer using a Nalorac dual tuned ^{1}H–^{19}F probe equipped with Z axis gradients. Samples for heteronuclear single quantum coherence (HSQC) spectra were prepared by simultaneous dialysis against a large volume of 20 mM sodium phosphate, pH 6.7, 20 μM EDTA, 0.01% NaN_3, 8% D_2O to ensure identical buffer conditions. Sample volumes of 500 μl were transferred to regular 5 mm NMR tubes for measurement. The protein concentration in the samples used for the natural abundance ^{1}H–^{13}C HSQC spectra was ~4 mM (WT), ~10 mM (WT labeled with 5F-Trp), ~16 mM (Y33F,Y45F mutant) and ~4 mM (Y33F,Y45F mutant labeled with 5F-Trp). Structural integrity of the proteins was assessed by ^{1}H–^{15}N HSQC spectra (at natural abundance for the fluorinated proteins; data not shown). ^{1}H–^{13}C HSQC spectra (only the aromatic region) and two-dimensional (2D) TOCSY and 2D NOESY (data not shown) experiments were recorded employing standard pulse sequences [9] using Watergate for H_2O suppression. The ^{1}H–^{19}F HOESY spectrum was collected using the pulse sequence of Rinaldi [10] which was modified to use the States–Ruben–Haberkorn method for data acquisition resulting in pure phase lineshapes in F_1. A pulsed field gradient of 20 G/cm and 2 ms duration was inserted into the mixing period just after the second proton 90° pulse to dephase any magnetization which was not inverted. Spectra were recorded with 3143 Hz spectral width and 512 complex points in t_2 (^{19}F) and 8000 Hz and 64×2 increments in t_1 (^{1}H) resulting in acquisition times of 77 ms and 8 ms, respectively. Ninety-six transients were recorded per t_1 increment and spectra were recorded with mixing times of 100 ms, 200 ms, 300 ms, 400 ms, 500 ms, 600 ms, 750 ms, 850 ms, 950 ms, 1100 ms and 2000 ms. ^{19}F spectra were referenced to TFA in D_2O. Assignments were obtained first for the wild-type protein, using the published proton assignments as starting points [1]. They were extended to the mutant spectra recorded under identical conditions. All spectra were processed with nmrPipe [11] and analyzed with nmrView [12].

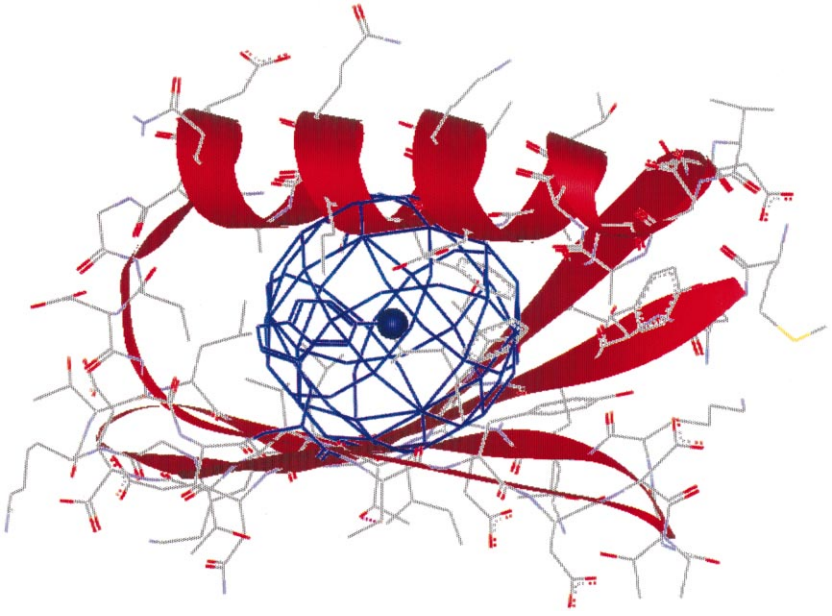

Fig. 1. Ribbon diagram of the B1 domain of streptococcal protein G. The single tryptophan residue (Trp43) is located on β-strand 3 and is partially buried in the core of the protein. The aromatic ring of the Trp side chain as well as a sphere of radius 6 Å around the F-atom at the ζ3 position are displayed in blue to illustrate its central location.

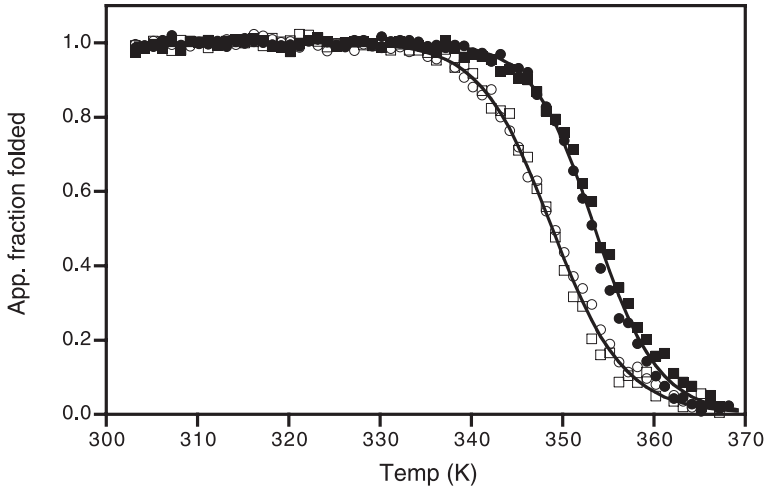

Fig. 2. Temperature-induced denaturation curves for wild-type GB1, 5F-Trp-labeled wild-type GB1 (●), wild-type GB1 protein labeled with 5F-Trp (■), the Y33F,Y45F mutant protein (○), and the Y33F,Y45F mutant protein labeled with 5F-Trp (□). The thermal unfolding curves were monitored by CD in 50 mM sodium acetate buffer, pH 5.5.

3. Results and discussion

We monitored the thermal unfolding transitions of wild-type GB1 and the Y33F,Y45F mutant GB1, with and without 5F-Trp labeling, by CD spectroscopy. The corresponding unfolding curves for all four proteins are displayed in Fig. 2. The melting temperature for wild-type GB1 at pH 5.5 is 80.5°C, similar to the values reported previously [1,13]. As can be appreciated, fluorination at the ζ3 position of the tryptophan side chain did not change the melting temperature at all. Likewise, although the mutant is clearly less stable than the wild-type GB1, no difference in melting temperature between the mutant and the fluorinated version was observed. Both proteins exhibit a melting temperature of 75.9°C. The fact that the mutant is ~5°C less stable than the wild-type protein is easily explained by the fact that substitution of Tyr45 by Phe

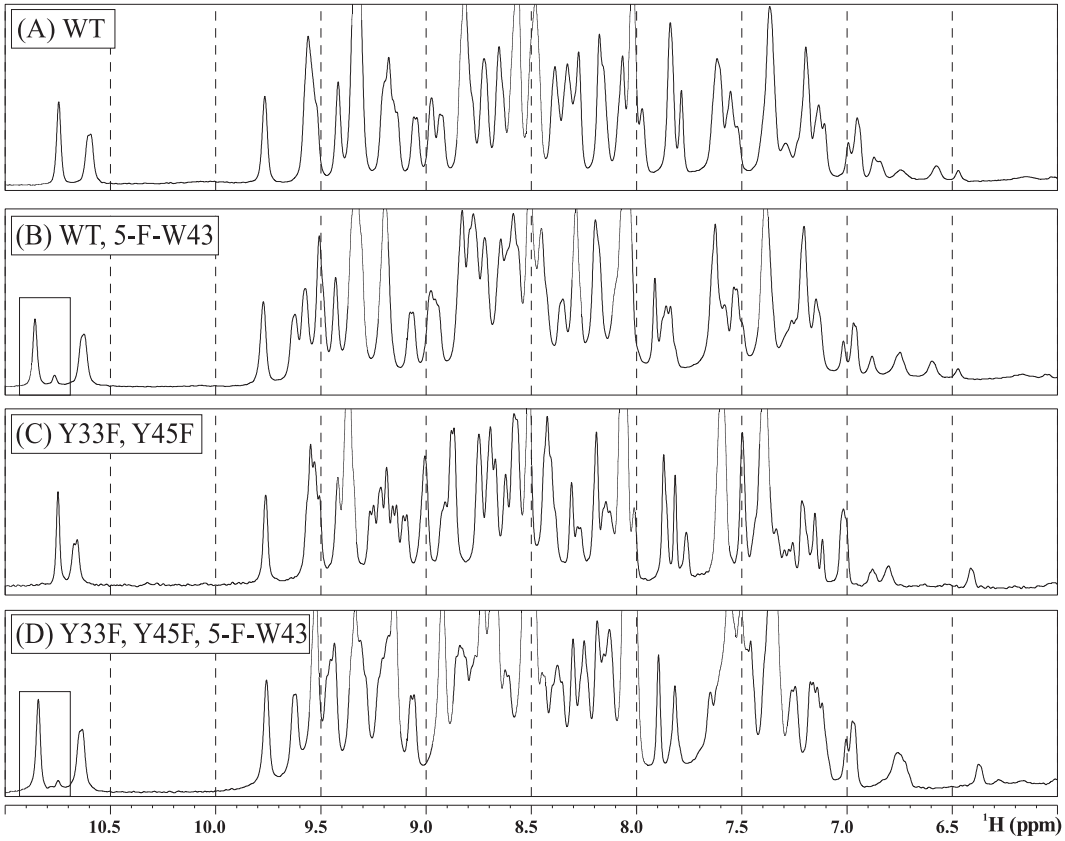

Fig. 3. Low field region of the 1D spectra of wild-type GB1 protein (A), wild-type GB1 protein labeled with 5F-Trp (B), the Y33F,Y45F mutant protein (C), and the Y33F,Y45F mutant protein labeled with 5F-Trp (D). The small residual Nε1H resonances (~5%) arising from unlabeled protein in (B) and (D) and their corresponding labeled signals (~95%) are boxed.

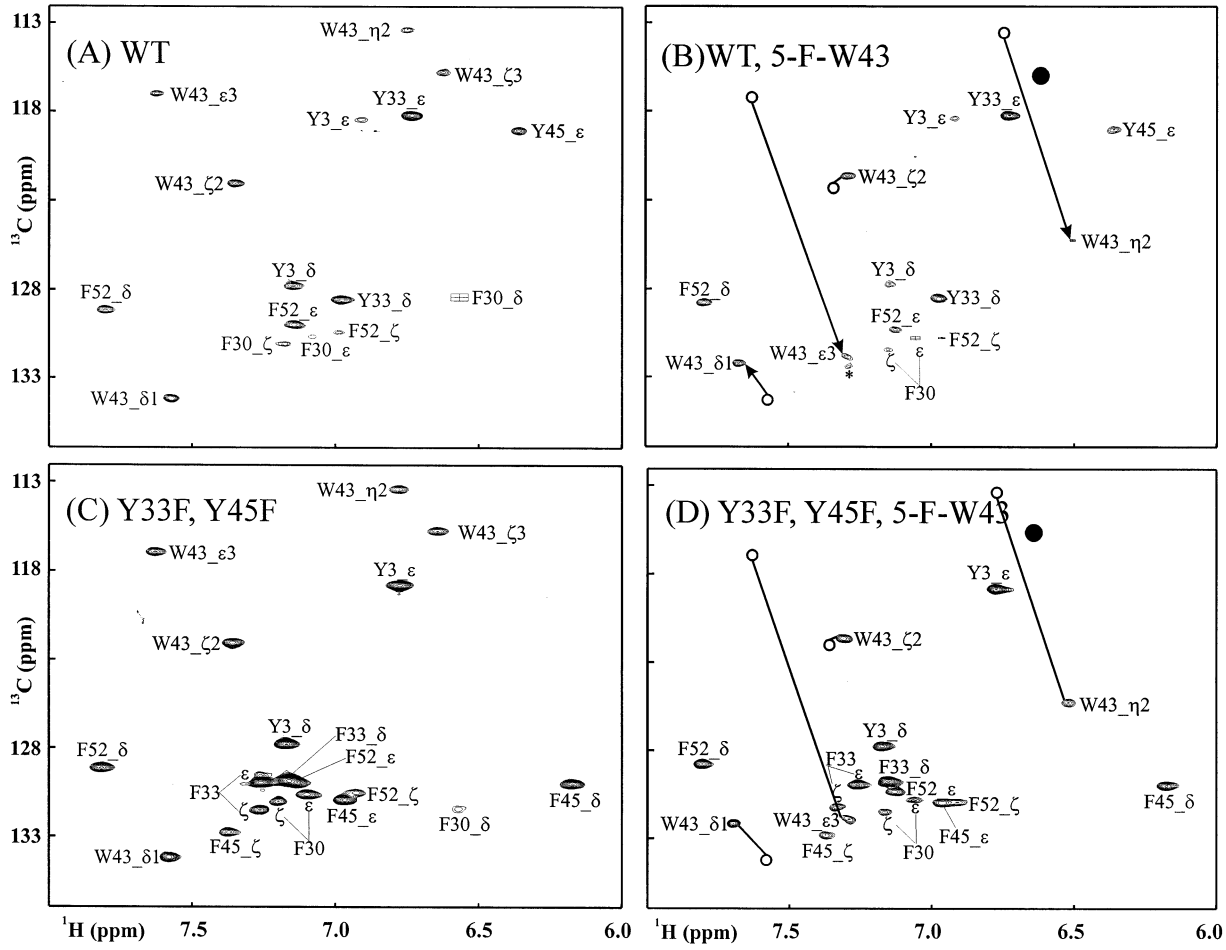

Fig. 4. Aromatic region of the ^{1}H–^{13}C HSQC spectra of wild-type GB1 protein (A), wild-type GB1 protein labeled with 5F-Trp (B), the Y33F,Y45F mutant protein (C), and the Y33F,Y45F mutant protein labeled with 5F-Trp (D). Assignments are indicated by residue type, number and atom identifier. Broad signals that do not appear at the contour levels displayed are represented with a crossed rectangle (e.g. F30_δ in (A)). The Y45_δ signal lies outside the spectral width displayed, and the asterisk indicates a signal of unknown origin. For spectra of the 5F-Trp-substituted proteins changes in position of the Trp43 aromatic signals (δ1, ζ2, η2, and ε3) compared to the corresponding non-fluorinated proteins are indicated by empty circles and arrows. The lost ζ3 signal upon fluorination is marked by a filled circle.

abolishes a hydrogen bond between the tyrosine hydroxyl and the carboxylate side chain of Asp47.

A comparison of the low field region of the one-dimensional (1D) NMR spectrum of all four proteins at 25°C is shown in Fig. 3. Only very small changes between the non-fluorinated and fluorinated proteins are observed. Most noticeable is the downfield shift of the indole Nε1H resonance, located at 10.75 ppm in the non-fluorinated proteins. For both 5F-Trp containing proteins a downfield shift of 0.09 ppm is observed, consistent with an increased shielding caused by introduction of a fluorine at the ζ3 position (David Case, personal communication). The downfield shift can be measured very accurately since ca. 5% of the protein remained non-fluorinated, as evidenced by a small signal at the original position (Fig. 3). Note that the amide proton resonance of Lys10 remains unaffected and resonates for all four proteins at ~9.75 ppm and that of Phe52 at 10.65 ppm varies only very little. These data suggested that no, or extremely small, changes in the three-dimensional (3D) structure of the protein occurred upon fluorination. Further evidence for this can be gleaned from the chemical shifts of the aromatic residues in the proteins. These amino acids reside in the hydrophobic core of GB1 and any structural rearrangements of the core would manifest themselves in chemical shift changes. The aromatic regions of the proton–carbon correlation spectra for all four proteins are displayed in Fig. 4. Comparison of the spectra recorded for the fluorinated proteins (B) and (D) with those of the corresponding non-fluorinated samples (A) and (C) demonstrates the loss of the Trp43ζ3 correlation (~6.6/~116 ppm) upon replacement of the proton by fluorine. In addition, substantial shifts of the Trp43ε3 and Trp43η2 correlations are observed. The Trp43ε3 cross-peaks move from ~7.6/~117 ppm to ~7.3/~131 ppm while those of Trp43η2 move from ~6.7/~113 ppm to ~6.5/~125 ppm. These shifts can be explained simply by the fact that ε3 and η2 are neighboring positions to the fluorine atom on the aromatic ring and both carbon and proton resonances consequently should exhibit the largest deshielding effects. The differences in carbon shifts are 12 and 15 ppm for the η2 and ε3 carbons, respectively, and are very similar to the shift differences observed for the free amino acid [14]. In contrast to the chemical shift changes caused by the altered chemical structure of the aromatic ring of fluorinated tryptophan, the other correlations are not, or only marginally, affected. For instance, resonances

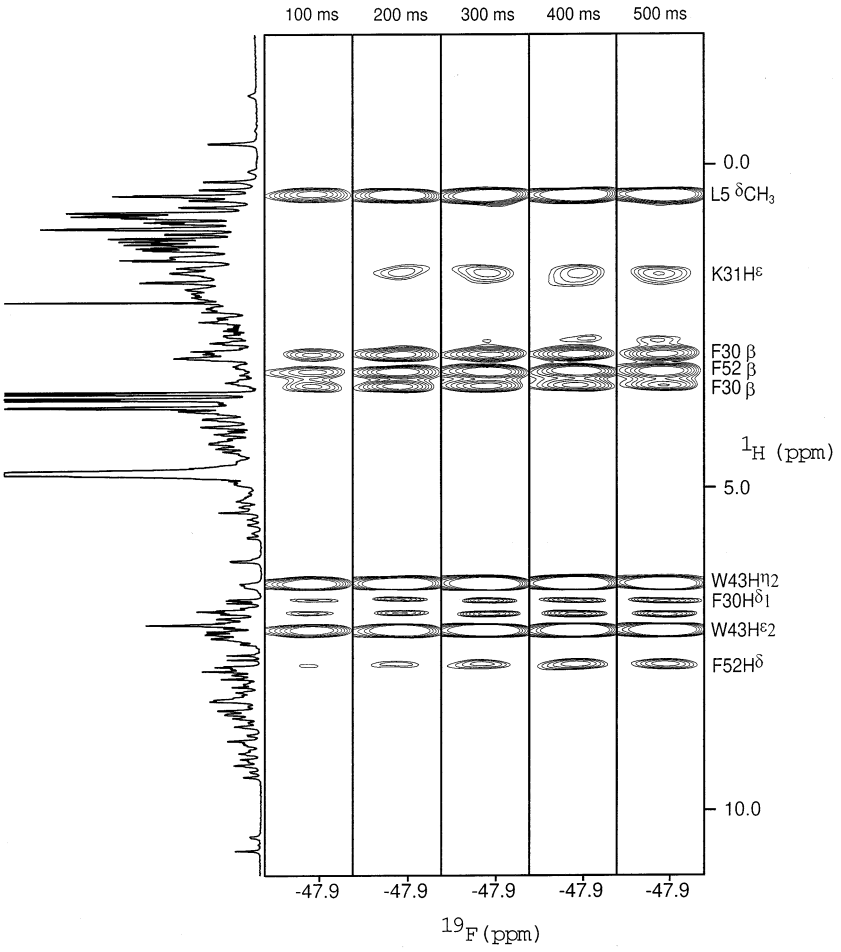

Fig. 5. 2D ^{1}H–^{19}F HOESY spectra of wild-type GB1 protein labeled with 5F-Trp recorded with different mixing times. The sample contained 1 mM protein in 20 mM phosphate buffer, pH 5.4. All spectral parameters were as described in Section 2. Assignments for all NOE cross-peaks are indicated by residue type, number and atom identifier on the right hand side of the panels.

arising from Tyr3 or Phe52 remain unchanged. Given the fact that in the wild-type structure the aromatic ring of Phe52 is located only 5 Å away from the Trp43ζ3 position, the lack of chemical shift changes upon introduction of the fluorine atom demonstrates unambiguously that the structure of the protein hydrophobic core of the F-Trp containing protein is very similar to the non-fluorinated one. Corresponding results and conclusions are valid for the mutant.

Fig. 5 displays strips from the 2D ^{19}F–^{1}H HOESY spectrum recorded on the 5F-Trp-labeled wild-type GB1 sample. Cross-peaks are observed between the F-atom and several aromatic ring protons. These are the ε3 (7.2 ppm) and η2 (6.5 ppm) protons of Trp43, which are separated from the ζ3 position by 2.5 Å. Inter-residue NOEs between the fluorine atom and the aromatic δ ring protons of Phe52, the β- or δ-methylene protons of Phe30, Phe52 and Lys31, and the Leu5 δ1 methyl group are observed as well. The distances between all these atoms and the fluorine vary between 2.5 and 4 Å, as judged by examination of the 3D structures of the non-fluorinated protein. Increasing the mixing time from 100 ms to 500 ms results in a linear intensity increase for these cross-peaks, as expected for the initial built-up regime. Note that we never observed any cross-peaks due to spin diffusion, even up to mixing times of 2 s. As can most clearly be observed for the Phe52δ and the Lys31ε cross-peaks, adjusting the time employed for magnetization transfer within the initial built-up regime, smaller or larger distance spheres around the fluorine atom can be mapped out. Indeed, these protons are located ~4.5–5 Å away from the F-atom.

In summary, we demonstrated that the protein 3D structure and thermal stability of the GB1 domain are not altered upon Trp fluorination at the ζ3 position. This is an intriguing observation, given the fact that the F-atom is positioned exactly at the center of the hydrophobic core in this protein. The benign nature of fluorination holds also true for the Y33F,Y45F mutant of GB1, which exhibits slightly reduced thermal stability compared to the wild-type protein, with both proteins melting at the same temperature. In this context it seems worth pointing out that NMR studies may prove to be superior to crystallographic ones in probing for the presence of structural changes, since subtle effects caused by fluorine substitution in proteins could be masked or counteracted by strong lattice forces in the crystal. Given that ^{19}F chemical shifts are dominated by effects of the lone pair electron and aromatic C–F bonds are highly polarizable, the present GB1 proteins constitute ideal model systems to investigate and monitor changes in H-bonding, electrostatic fields and van der Waals effects in the interior of the hydrophobic protein core. In addition, local motions within the GB1 core can be studied using fluorine relaxation.

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