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5-Fluorotryptophan as a Dual NMR and Fluorescent Probe of α -Synuclein

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i. Summary

Analysis of conventional proton nuclear magnetic resonance (NMR) experiments on intrinsically disordered proteins (IDPs) is challenging because of the highly flexible and multiple rapidly exchanging conformations typifying this class of proteins. One method to circumvent some of these difficulties is to incorporate non-native fluorine (^{19}F) nuclei at specific sites within the polypeptide. ^{19}F NMR is particularly suitable for characterization of unfolded structures because ^{19}F chemical shifts are highly sensitive to local environments and conformations. Furthermore, the incorporation of fluorine analogs of fluorescent amino acids such as 5-fluoro-tryptophan (5FW) allows for complementary studies of protein microenvironment *via* fluorescence spectroscopy. Herein we describe methods to produce, purify, characterize, and perform steady-state fluorescence and 1-D NMR experiments on 5FW analogues of the IDP α -synuclein.

Keywords

Fluorine; Parkinson's Disease

1. Introduction

NMR spectroscopy is an indispensable tool for protein structure determination. While well-folded proteins generally exhibit distinct proton (^1H) NMR resonances enabling assignment to specific nuclei, the innate flexibility and multiple conformations associated with intrinsically disordered proteins (IDPs) make assignments using conventional proton NMR methods a challenge (1). In these cases, performing NMR on IDP analogues that contain a fluorine (^{19}F) nucleus is particularly useful. ^{19}F NMR has several advantages over ^1H NMR and requires no additional technology (^{19}F spin = $\frac{1}{2}$ nucleus). For example, ^{19}F resonances are exquisitely sensitive to changes in local environment with 9 as opposed to 1 electron surrounding the fluorine nucleus resulting in a greatly enhanced chemical shift dynamic range for ^{19}F (up to 400 ppm) as compared to ^1H (up to 13 ppm) (2–5). Furthermore, with advancements in biosynthetic methods for incorporation of fluorinated amino acids in proteins during bacterial growth (6), fluorine NMR can be applied to study a wide variety of previously difficult, large proteins and IDPs (3, 7, 8). A good candidate is the fluorinated tryptophan analog, 5-fluoro-tryptophan (5FW). Because Trp is typically in low abundance in most polypeptide sequences, minimal site-directed mutagenesis is required to make specific

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¹⁹ Generally ~15 μL of 50 – 100 μM α -syn is sufficient to perform ESI-MS. Speak with your core facility manager for specific requirements and procedures. Expected molecular weights for fluorinated α -syn variants are 14,469 for 5FW4/Y125F/Y133F/Y136F and 5FW94/Y125F/Y133F/Y136F, and 14,451 for 5FW39/Y125F/Y133F/Y136F.

resonance assignments and most importantly, fluorescence spectroscopy can be employed as an additional site-specific probe of local environments and conformation.

In this chapter, we present methods to produce, purify, and characterize using ^{19}F NMR and fluorescence spectroscopy single 5FW mutants of an example IDP α -synuclein (α -syn). While several other fluorine containing amino acid analogs have been used in ^{19}F studies of α -syn (7, 9–11), we choose 5FW for its utility as a dual NMR and fluorescence probe (*vide supra*) (8). Specifically, we describe procedures to express 5FW-containing proteins in *Escherichia coli* by inhibiting the Shikimate pathway for aromatic amino acid synthesis with N-phosphonomethyl-glycine (glyphosate) (6). The importance of SDS-PAGE, UV-Vis spectroscopy, and ESI-MS in assessing protein purity is also emphasized. Furthermore, to highlight the utility of 5FW as a dual NMR and fluorescence probe of the local microenvironment and protein conformation of α -syn, experimental protocols for both techniques are presented.

2. Materials

2.1 Production of 5-Fluorotryptophan-containing Proteins

1. QuickChange kit for site-directed mutagenesis (Statagene)
2. Primers for α -syn single tryptophan variants
3. α -Syn expression plasmid (pRK172) (12)
4. DNA sequencing service
5. BL-21(DE3)pLysS *Escherichia coli* cells
6. Heat Block (42°C)
7. SOC medium
8. Antibiotic stocks: chloramphenicol (CM, 34 mg/mL) and ampicillin (AMP, 100 mg/mL)
9. Lauria Broth (LB) agar: tryptone (16 g/L), NaCl (16 g/L), yeast extract (8 g/L), bacto agar (24 g/L)
10. Cell culture plates (100 × 15 mm style)
11. Cell spreader
12. Sterile toothpick
13. Lauria Broth (LB) Medium: tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L), pH 7.0)
14. Temperature controlled incubator shaker
15. Centrifuge tubes (30 mL – 1 L)
16. Centrifuges for 30 mL – 1 L centrifuge tubes
17. 5 mL sterile pipettes
18. Disposable 1 cm path length cuvettes rated for visible spectroscopy
19. N-(Phosphonomethyl)glycine (glyphosate)
20. 5-fluoro-D,L-tryptophan (5FW)
21. Modified New Minimal Medium (NMM) (8, 13): ammonium chloride (7.5 mM), NaCl (8.5 mM), potassium dihydrogenphosphate (55 mM), potassium

hydrogenphosphate (100 mM), magnesium sulfate (1 mM), glucose (20 mM), Ca^{2+} (1 mg/L), Fe^{2+} (1 mg/L), Zn^{2+} (1 $\mu\text{g/L}$), thiamine (10 mg/L), uracil (10 mg/L), all amino acids except tryptophan and proline (40 mg/L) (*see* Note 1)

22. Glucose (1 M)
23. Sterile erlenmeyer flasks
24. UV-Vis spectrometer
25. Wash Medium: ammonium chloride (7.5 mM), NaCl (8.5 mM), potassium dihydrogenphosphate (55 mM), potassium hydrogenphosphate (100 mM), magnesium sulfate (1 mM), glucose (20 mM)
26. 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG)

2.2 Protein Purification

1. Lysis Buffer: 100mM Tris, 300mM NaCl, 1mM EDTA (pH 8.2)
2. 100 mM phenylmethylsulfonyl fluoride (PMSF) (*see* Note 2)
3. Bench top probe homogenizer
4. Stirring hot plate
5. pH meter with calomel pH probe
6. Concentrated HCl
7. Dialysis buffer: 20 mM Tris, 1 mM EDTA (pH 8.0)
8. Dialysis tubing with <10 kD molecular weight cut off (*see* Note 3)
9. Dialysis tubing closures
10. Chromatography buffers: 20 mM Tris and 20 mM Tris, 1M NaCl (pH 8.0) (*see* Note 4)
11. Fast protein liquid chromatography (FPLC) system equipped with fraction collector and a UV (280 nm) detector
12. HiPrep DEAE FF and MonoQ anionic exchange columns (GE Healthcare)
13. Gel electrophoresis machine equipped with gel developer
14. UV-Vis spectrometer
15. 1 cm path length quartz cuvette for absorption
16. Mass spectrometry facility with electrospray ionization mass spectrometer (ESI-MS)

¹ NMM media is prepared as the following. Autoclave 0.9 L water in 4 L erlenmeyer flasks and add other reagents as described. Prepare 10X salt solution (KH_2PO_4 0.55M; K_2HPO_4 1M; NaCl 85 mM) adjust pH to 7.2 with NaOH, filter sterilize, and add 100 mL to autoclaved water. Autoclave individual stocks of ammonium chloride (1 M), magnesium sulfate (1 M), and glucose (1 M) and add to a final concentration of 7.5, 1, and 20 mM, respectively. Stocks of Ca^{2+} (1 g/L) and Fe^{2+} (1 g/L) and Zn^{2+} (1 mg/L) were filter sterilized and added to a final concentration of 1 mg/L for Ca^{2+} , Fe^{2+} , and 1 $\mu\text{g/L}$ for Zn^{2+} . Stocks of thiamine (2 g/L) and uracil (10 g/L) were filter sterilized and added to a final concentration of 10 mg/L. All amino acids except tryptophan and proline (40 mg/L each) were weighed, premixed, added as a solid, and shaken well (0.72 g per L media).

² A fresh stock solution of PMSF (100 mM) should be prepared in ethanol or isopropanol daily and kept at 4°C until use. PMSF, a serine protease inhibitor, is a cytotoxic chemical and should be handled with gloves at all times and disposed appropriately.

³ Some dialysis tubing must be treated prior to use. Please follow instructions from the dialysis tubing manufacturer for specific details.

⁴ Buffers should be filtered through a 0.22 μm pore size filter prior to use.

17. Protein concentration device with molecular weight cutoff < 10 kD

2.5 Spectroscopic Characterization of ¹⁹F α-synucleins

1. PD-10 desalting columns (GE Healthcare)
2. Buffer for spectroscopy: 10 mM sodium phosphate, 100 mM NaCl (pH 7.4) (*see* Note 4)
3. N-acetyl-tryptophanamide (NATA)
4. 5-fluoro-D,L-tryptophan (5FW)
5. YM-100 centrifugal filter units
6. Bench-top ultracentrifuge
7. UV-Vis spectrometer
8. Spectrofluorometer equipped with temperature controlled cuvette holder
9. 1 cm path length quartz cuvette for fluorescence

2.4 ¹⁹F NMR

1. NMR spectrometer
2. D₂O
3. Trifluoroacetic acid
4. Data processing software

3. Methods

3.1 Production of 5-Fluorotryptophan Proteins

1. Since WT α-syn does not contain any native Trp residues, site-directed mutagenesis must be performed to produce single Trp-containing α-syn plasmids at native aromatic sites (F4W, Y39W, F94W). This can be done using the QuickChange kit, the wild type α-syn expression plasmid (pRK172) (12), and appropriate PCR primers (*see* Note 5).
2. Confirm desired mutations by DNA sequencing.
3. Transform BL21(DE3)pLysS *Escherichia coli* cells with appropriate pRK172 constructs by gently pipetting 1 μL DNA into 25 μL BL21(DE3)pLysS cells. Incubate tube containing DNA/cell mixture on ice for 15 min and then place into heat block at 42°C for 80 seconds followed by incubation on ice for 2 minutes. Add 200 μL SOC medium (*see* Note 6).
4. Pipette 15 μL of transformed cells onto LB agar cell culture plates containing CM (34 μg/mL) and AMP (100 μg/mL) antibiotics. Uniformly spread cells using a sterile cell spreader (*see* Note 7).

⁵ A detailed protocol for performing site-directed mutagenesis reactions and designing appropriate primers can be found in the QuickChange kit.

⁶ If using small tubes (0.5 mL) expose DNA/cells to heat block for 40 s.

⁷ LB agar plates containing appropriate antibiotics can be made by diluting stocks of CM (68 mg/mL) and AMP (100 mg/mL) to final concentrations of 34 μg/mL and 100 μg/mL, respectively into presterilized LB agar medium. If using an autoclave to sterilize LB, first let media cool such that is warm to the touch prior to adding antibiotics. Pour warm LB with antibiotics into cell culture plates (100 × 50 mm size) until cell culture plates are approximately half full. Cover plates and let cool to room temperature before use. Plates can be stored for ~1 month at 4°C for future use.

5. Incubate cell culture plates at 37°C for 8–12 hours or until distinct cell colonies are observed.
6. Using a sterilized toothpick, inoculate a sterilized 125 mL Erlenmeyer flask containing 25 mL of LB media, CM (34 µg/mL), and AMP (100 µg/mL). Incubate for 8–12 hours at 37°C shaking at 170 RPM.
7. Pour cells into a sterile 50 mL centrifuge tube and centrifuge at $2900 \times g$ (4°C) for 10 min.
8. Pour off supernatant and re-suspend pellet with 20 mL LB containing CM (34 µg/mL) and AMP (100 µg/mL).
9. Add re-suspended pellet to a sterilized 4 L Erlenmeyer flask containing 1 L LB media containing CM (34 µg/mL), AMP (100 µg/mL), and glucose (20 mM).
10. Incubate 1 L culture at 30°C (shaking 170 RPM) until the optical density (O.D.) at 600 nm $\approx$ 0.6 – 0.8 (*see* Note 8).
11. Pour cell culture into pre sterilized centrifuge tubes and centrifuge at $5000 \times g$ (4°C) for 30 min.
12. Wash harvested cells with Wash medium (2.1.25).
13. Resuspend harvested cells in 20 mL of media (2.1.21) and then use 10 mL to inoculate each 1 L media containing glyphosate (1 g/L), 5FW (40 mg/mL), CM (34 µg/mL), and AMP (100 µg/mL) in 4 L sterilized erlenmeyer flask.
14. Incubate culture at 30°C (shaking 170 RPM) for 30 min.
15. Induce protein expression by adding IPTG to a total concentration of 0.5 – 1 mM.
16. Continue incubating at 30°C (170 RPM) for 5 – 6 hours.
17. Pour cell culture into centrifuge tubes and centrifuge at $5000 \times g$ (4°C) for 30 min.
18. Remove supernatant and collect cell pellet (*see* Note 9).

3.2 Protein Purification

1. Prepare a boiling water bath (*see* Note 10).
2. Resuspend 2 L pellet in 50 mL lysis buffer (2.2.1; 25 mL per 1 L culture) with freshly added PMSF (0.5 mM).
3. In medium size (150 mL) centrifuge bottle, homogenize pellet using a bench top probe homogenizer (*see* Note 11).
4. Put small stir bar in tube with homogenate and purge with gentle nitrogen stream for 15 minutes at room temperature.
5. Seal bottle and place in boiling water bath for 15 minutes.
6. Centrifuge sample for 30min at $20,000 \times g$ (4°C).
7. Collect supernatant in beaker with stir bar.

⁸ To check O.D., remove ~1mL of culture from erlenmeyer flask using a sterile 5 mL pipette and place into a disposable cuvette. Place ~1 mL of culture media that is not inoculated in a separate cuvette. Use a spectrometer to measure O.D. at 600 nm for media alone. Repeat for inoculated media. Subtract O.D. of inoculated media from that of media alone. If O.D. of inoculated media is between 0.6 and 0.8, proceed to step 3.1.10. If O.D. is < 0.6 continue to incubate culture and retest O.D. as needed.

⁹ Pellet can be used immediately or stored at –20°C for later use.

¹⁰ To prepare the boiling water bath, place a 1 L beaker filled $\frac{3}{4}$ with water and a stir bar on a stirring hot plate.

¹¹ While thorough homogenization is essential and no clumps should be visible, avoid excess homogenization resulting in foam.

8. While stirring on a stir plate, add concentrated HCl dropwise until pH reaches 3.5 (*see* Note 12).
9. Place beaker with sample on stir plate for 15 min at 4°C
10. Place sample in centrifuge tubes and centrifuge at $20,000 \times g$ (4°C) for 1h.
11. Collect supernatant.
12. Prepare two 4 L beakers of dialysis buffer (2.2.7) and add a large stir bar to each beaker.
13. Place sample into dialysis tubing and carefully clamp each tube end with dialysis tubing closures.
14. Add fresh PMSF to a total concentration of 0.5 mM to the first beaker containing dialysis buffer and gently place sample in dialysis tubing into beaker. Incubate for 6–12 hours on stir plate at 4°C.
15. Add freshly prepared PMSF to 0.5 mM to the 2nd beaker of dialysis buffer. Remove sample from first dialysis beaker and gently place in the 2nd beaker. Incubate for 6–12 hours on stir plate at 4°C.
16. Collect sample in small beaker and load onto a pre-equilibrated (20 mM Tris, pH 8.0) HiPrep 16/10 DEAE FF anionic exchange column (*see* Note 13).
17. Elute protein using a linear gradient of 20 mM Tris and 1 M NaCl. Collect 8 mL fractions of eluent. α -Syn protein will elute upon exposure to 100 – 300 mM NaCl (*see* Note 14).
18. Evaluate fractions corresponding to peaks in the O.D. at 280 nm by SDS-PAGE (*see* Note 15).
19. Evaluate fractions corresponding to peaks in the O.D. at 280 nm by UV-Vis spectroscopy (*see* Note 16).
20. Combine fractions containing pure or semi-pure α -syn and concentrate using a protein concentration device with a MWCO < 10 kD.
21. Exchange buffer *via* dialysis according to steps 13 – 15.
22. Load sample onto a pre-equilibrated (20 mM Tris, pH 8.0) MonoQ anionic exchange column.
23. Elute, evaluate, and concentrate purified α -syn protein according to steps 17 – 20. An example MonoQ chromatogram is shown in Figure 1. Purified α -syn corresponds to the large singular peak between 425 – 500 mL with O.D. at 280 nm

¹² As the solution pH will change dramatically with the addition of each drop of concentrated HCl make sure pH has completely stabilized before adding additional drops. At pH 3.5, a significant amount of protein will have visibly precipitated.

¹³ Refer to FPLC manual and chromatography column information for specific details regarding proper column equilibration and general use.

¹⁴ Make sure FPLC is equipped to monitor and record the O.D. of eluted sample at 280 nm. Fractions containing α -syn protein can be identified by a peak in the O.D. at 280 nm.

¹⁵ Sample from each fraction thought to contain α -syn protein should be analyzed on an SDS-PAGE gel. Protein corresponding to α -syn monomer will run near ~15,000 kD. Use of a silver staining method for gel development will ensure all fractions containing a small amount of α -syn will be visible. Combine pure protein for use (only α -syn band is observed) or combine semi-pure α -syn fractions (multiple protein bands including α -syn are observed) for further purification.

¹⁶ A UV-Vis spectrum should be collected for sample from each fraction thought to contain α -syn protein. The UV-Vis spectra should be collected between 200 – 500 nm using a 1 cm pathlength quartz cuvette. Purified proteins will exhibit a single maximum peak in O.D. around 280 nm with a pronounced right shoulder corresponding to the presence of 5FW. If fractions contain DNA or other contaminants, additional peaks will be observed. Combine pure protein for use (only single α -syn peak observed) or combine semi-pure α -syn fractions (peaks for both α -syn and other contaminants are observed) for further purification.

greater than that of other wavelengths (250 and 290 nm) recorded. α -Syn elutes between 100 – 300 mM NaCl corresponding to ~10 – 30 % Buffer B (20 mM Tris, 1 M NaCl) (*see* Note 17).

24. Evaluate protein purity using SDS-PAGE.
25. Combine pure fractions and concentrate to desired volume.
26. Use UV-Vis absorbance spectroscopy to determine the concentration of purified protein (*see* Note 18).
27. Confirm appropriate molecular weight of protein by performing electrospray ionization mass spectrometry (ESI-MS) on purified protein.
28. Use protein right away for experiments or flash freeze and store at -80°C for later use (*see* Note 20).

3.3 Spectroscopic Characterization of ^{19}F containing synucleins

1. Use a PD-10 column to buffer exchange up to 2.5 mL purified protein into the buffer for spectroscopy (10 mM NaPi, 100 mM NaCl buffer, pH 7.4).
2. Determine the concentration of desalted protein using UV-Vis spectroscopy (*see* Note 18).
3. Prepare a sample of 10 μM fluorinated protein by diluting the concentrated protein stock with the buffer for spectroscopy.
4. Prepare a 10 μM sample of the tryptophan model complex N-acetyl-tryptophanamide (NATA) using the buffer for spectroscopy. The molar extinction coefficient at 280 nm for NATA is $5500 \text{ M}^{-1} \text{ cm}^{-1}$.
5. Prepare a 10 μM sample of the 5FW using the buffer for spectroscopy. The molar extinction coefficient at 280 nm for 5FW is $5700 \text{ M}^{-1} \text{ cm}^{-1}$.
6. Take a UV-Vis spectrum of all 10 μM samples (200 – 500 nm).
7. Substitution of the fluorine atom on the tryptophan indole ring causes a characteristic red shift in the absorption spectrum as compared to native tryptophan. This shift (~5 nm) is most obvious upon comparison of the red edge of the NATA and 5FW absorption traces (*see* Figure 2). To confirm high incorporation of the 5FW into α -syn protein, compare of the red edge of the UV-Vis absorption spectrum of fluorinated protein sample to that of both NATA and 5-fluorotryptophan in buffer alone. If >90% of the protein has 5-fluorotryptophan incorporated, the absorption of the protein sample should overlay with that of 5-fluorotryptophan in buffer alone.
8. To evaluate the local environment of 5-fluorotryptophan incorporated in α -syn protein samples, collect a fluorescence emission spectra by placing a fluorescence

¹⁷ If there are many peaks in O.D at 280 nm corresponding to the α -syn containing region (100 – 300 mM NaCl) after purification using both the 16/10 HiPrep DEAE FF and MonoQ columns, repeat steps 3.2.20 – 3.2.23 until the MonoQ chromatogram resembles Figure 1 and SDS-PAGE gels and UV-Vis spectra reflect >95% purity.

¹⁸ If using a 1 cm pathlength quartz cuvette, the concentration of protein can be calculated according to Beer's Law by dividing the O.D. at 280 nm by the estimated molar extinction coefficient at 280 nm ($\epsilon_{280\text{nm}}$). For 5FW4 and 5FW94 proteins $\epsilon_{280\text{nm}} = 6980 \text{ M}^{-1}\text{cm}^{-1}$ and for 5FW39 protein $\epsilon_{280\text{nm}} = 5700 \text{ M}^{-1}\text{cm}^{-1}$ (in these variants C-terminal Tyr had been mutated to Phe). When calculating protein concentrations make sure there is no contribution from buffer background. To do this, subtract the O.D. of buffer alone at 280 nm from that of the protein.

²⁰ To flash freeze, place liquid nitrogen in a small dewar and drop tubes containing protein into the dewar. Once protein samples have frozen, remove carefully with tweezers and place in -80°C freezer.

quartz cuvette containing sample in a temperature controlled spectrofluorometer cuvette holder (*see* Note 21).

9. Set spectrofluorometer to the following parameters: T = 25°C, excitation = 295 nm; slit width 1 nm, emission = 300 – 500 nm; slit width = 1 nm, integration time = 0.25 s (*see* Note 22).
10. Collect the steady-state emission spectrum for all protein samples and buffer. The emission spectra for NATA and 5FW alone in spectroscopy buffer are displayed in Figure 2 (*see* Note 23).

3.4 ¹⁹F NMR

1. Desalt protein samples and determine protein concentration according to 3.3.1 – 3.3.2.
2. Prepare 500 µL samples of 50 µM fluorinated protein samples by diluting the concentrated protein stock with buffer for spectroscopy and 50 µL D₂O (10% v/v).
3. Place samples into appropriate NMR tubes and then into the NMR sample chamber holder.
4. Use trifluoroacetic acid as an external standard (0 ppm).
5. Set NMR spectrometer to collect spectra in 1500 transient blocks over 2 – 11 h at 15°C with the following parameters: relaxation delay of 1.0 s, pulse width of 7.6 µs, and sweep width of 20 kHz.
6. Collect data for all fluorinated proteins and 50 µM 5FW alone according to 3.4.1 – 3.4.5.
7. Use data processing software to analyze data.
8. Evaluate the local environment of 5FW at each of the sites in α-syn tested by comparing the ¹⁹F NMR spectra of fluorinated α-syn variants to that of 5FW in buffer alone. The ¹⁹F NMR spectra of fluorine containing α-synucleins and free 5FW are given in Figure 3. The ¹⁹F NMR spectra reveal that all α-synucleins exhibit ¹⁹F resonances similar to the model complex (5FW) confirming the absence of secondary and tertiary structure for this intrinsically disordered protein. Though modest, small differences between the ¹⁹F resonances at the different sites reflect minor differences in the three protein microenvironments.

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²¹ If using a small volume quartz cuvette, make sure that the excitation beam is aligned to propagate through the 1 cm pathlength with adjacent quartz windows aligned parallel to fluorescence emission detector.

²² Slit widths and integration time may need to be adjusted to ensure adequate signal detection depending on the specific spectrofluorometer used.

²³ The spectrofluorometer manual should be consulted to ensure that measured counts are within the linear range of the detector. For all measurements the O.D. of the sample should be no greater than 0.1 at the excitation wavelength. For all samples, a buffer blank spectrum should be recorded to ensure that no contaminants are contributing to the emission spectrum. The 5FW emission spectrum in Figure 2 represents a solvent exposed chromophore. The spectral properties (quantum yield and wavelength of maximum emission) will change if the 5FW microenvironment changes.

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Figure 1.

Purification of α -synuclein (α -syn) using a MonoQ anionic exchange column. The elution of α -syn protein from a MonoQ column is shown with absorbance at 280, 250, and 290 nm denoted by red, blue, and pink traces, respectively. The green trace represents the linear gradient of buffer B (20 mM Tris, 1 M NaCl, pH 8).

Figure 2. Absorption and emission spectra of 10 μ M tryptophan model complex, NATA, and 5FW (20 mM sodium phosphate buffer, pH 7.4, 25°C). Inset: Chemical structure of 5FW.

Figure 3.

The 470 MHz ^{19}F NMR spectra of free 5FW and 5FW containing α -synucleins. (Top) Schematic representation of the α -syn primary sequence with 5FW mutations highlighted. (Bottom) Single ^{19}F resonances for all three synuclein variants (-48.9 ppm (5FW4), -49.1 ppm (5FW39), -49.1 ppm (5FW94)) and the model complex (-49.0 ppm). All spectra were collected at 15°C to avoid protein aggregation.