Small, but powerful and attractive: ^{19}F in biomolecular NMR

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Running title: *The power of Fluorine NMR*

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy is a versatile tool for probing structure, dynamics, folding and interactions at atomic resolution. While naturally occurring magnetically active isotopes, such as ^{1}H, ^{13}C, ^{15}N, are most commonly used in biomolecular NMR, with ^{15}N and ^{13}C isotopic labeling routinely employed at the present time, ^{19}F is a very attractive and sensitive alternative nucleus, which offers rich information on biomolecules in solution and in the solid state. This Persective summarizes the unique benefits of solution and solid state ^{19}F NMR spectroscopy for the study of biological systems. Particular focus is on the most recent studies and on future unique and important potential applications of fluorine NMR methodology.

Introduction

Understanding living systems builds on the enumeration of molecular structures and characterization of their dynamic interplay, as well as the inherent overall architecture and temporal rearrangements within the object under investigation. The complexity of biology poses particular challenges for methods designed to interrogate such systems, particularly with respect to sensitivity, specificity, information content, as well as spatial and time resolution. A given methodological tool may be suitable to investigate some entities and answer a particular question but may not be useful for others. Highly sensitive methods, such as single-molecule fluorescence spectroscopy (Moerner and Fromm, 2003), permit the investigation of very small amounts or low concentration samples but provide only a single observable for the entire object that may contain thousands of atoms.

Atomic-detail structures of biological macromolecules are most commonly determined *in vitro* by employing a divide-and-conquer strategy. This approach has been highly successful over the last 50+ years, with X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and cryo electron microscopy (cryoEM) having resulted in ~159,503 ~13,466 and ~8,510 structures, respectively, available from the PDB as of Sept. 5, 2021 (rcsb.org (Berman et al., 2000)). Each of these methods targets distinct properties of the molecules: electrons diffract X-rays, and the diffraction patterns can be transformed into atomic positions; nuclear spins give rise to the resonance frequencies in NMR, and magnetization transfers between spins can be converted into interatomic distances and angles; and, in cryoEM, elastically scattered electrons allow reconstruction of the object/molecular entity that is under investigation. As such, these complementary techniques have found their unique spaces in which they can provide indispensable information for guiding drug design, evaluating evolutionary relationships, inferring functional properties or guiding synthetic biology approaches. Indeed, integrated structural biology has been embraced by most practitioners as the optimal way to go forward(Gronenborn, 2019; van den Bedem and Fraser, 2015; Ward et al., 2013).

Commonly used isotopes in NMR

For biomolecular NMR applications, a prerequisite for any study is that the molecule, such as a protein, nucleic acid, lipid, carbohydrate, or complexes thereof, contains suitable 'NMR active' isotopes. At present, the most common and relevant isotopes are ^{1}H, ^{13}C, ^{15}N and ^{31}P, and uniform or site-specific labelling with ^{13}C and ^{15}N is generally carried out. Unlike these isotopes, which are exploited in all modern 3- and 4-D heteronuclear NMR techniques, fluorine or, specifically, the 100%-abundant magnetically active ^{19}F isotope, has been less common as an NMR probe, despite its unique attractiveness and early recognition of its potential (Gerig, 1994; Luck and Falke, 1991; Sykes and Hull, 1978). The high gyromagnetic ratio endows ^{19}F with excellent sensitivity (83% of ^{1}H, Table 1) and because the shielding of the ^{19}F nucleus is dominated by a large paramagnetic term, ^{19}F NMR parameters, such as chemical shifts and scalar coupling constants, are exquisitely responsive to local conformational and electronic environments. This is vividly obvious by simply inspecting the ^{19}F spectra of fluorinated Trp, either for the isolated amino acid or when incorporated into a protein (Figure 1). Unfortunately, there is still no general theoretical basis for predicting fluorine chemical shifts in proteins, as well as the effects of fluorination on protein structure. Fluorine exerts complex electronic effects, such as hyperconjugation, however to what extent this is influenced by the local environments and vice-versa is unclear. In order to make progress towards a more thorough understanding, we need to populate a database of fluorinated protein structures alongside the experimentally measured ^{19}F chemical shifts, interfaced with theory-based computational efforts.

Properties of Fluorine

Fluorine is a very small atom, the lightest member of the halogens, and the most reactive chemical element because of its extreme ability to attract electrons. Its toxic properties and difficulty in handling contributed to the slow progress in fluorine chemistry up to the 1920s. At the present time, fluorine incorporation into chemical moieties has been widely applied, and specific uses of fluorinated molecules have been discussed by many research groups in the last 20 years (Bégué and Bonnet-Delpon, 2007; Bohm et al., 2004; Gillis et al., 2015; Hagmann, 2008; Meanwell et al., 2014; Muller et al., 2007; Purser et al., 2008; Shah and Westwell, 2007; Wang et al., 2014; Yamazaki et al., 2009). Fluorine chemistry has gained a particularly large footprint in medicinal chemistry and drug discovery (Clayden, 2019; Richardson, 2016). Fluorine is frequently substituted for hydrogen or oxygen atoms since the van der Waals radius of the ^{19}F atom (1.47 Å) lies between that of a hydrogen (1.2 Å) and oxygen (1.52 Å) Table 1, and the bond length of C–F (1.41 Å) is similar to C–O (1.43 Å) and somewhat larger than to C–H (1.09 Å). Generally, C–F, C–OH and C–OMe are considered bioisosteric, and strategic substitution of F atoms for hydrogens, hydroxyl groups, or carbonyl oxygens is generally considered weakly perturbing, albeit this needs careful assessment for each individual substitution. For proteins, a recent report describes a careful evaluation of the effects of different levels and types of fluorination in the *Bacillus subtilis* cold shock protein (*Bs*CspB) (Welte et al., 2020).

Another unique and important consideration for biological applications is the essentially complete absence of fluorine in biology. Yet, because it can be incorporated synthetically or biosynthetically into most classes of biologically important molecules, such as lipids (Gagnon et al., 2018), carbohydrates (Linclau et al., 2020; Matei et al., 2013; Tsuchiya, 1990), proteins (Sharaf and Gronenborn, 2015) and nucleic acids (Chu and Horowitz, 1989; Morihiro et al., 2017), fluorine atoms can function as unique and easily identifiable NMR beacons in large and complex biological systems. In addition, fluorinated molecules can be investigated in any routinely used buffer system or environment without suffering from interference by background signals from natural abundance molecules.

^{19}F-modified proteins

Methods to prepare ^{19}F-modified proteins fall into three main categories: (i) post-translational covalent attachment of ^{19}F-containing moieties to the protein (Klein-Seetharaman et al., 1999), (ii) biosynthetic amino acid type-specific incorporation of ^{19}F-containing amino acids (Campos-Olivas et al., 2002; Crowley et al., 2012; Frieden et al., 2004; Sykes and Hull, 1978) and (iii) site-specific incorporation of ^{19}F-modified amino acids using recombinantly expressed orthogonal amber tRNA/tRNA synthetase pairs (Cellitti et al., 2008; Noren et al., 1989; Peeler and Mehl, 2012; Sharaf et al., 2016) (Figure 2).

Most covalent modifications introduce ^{19}F atoms into a protein of interest by conjugating a ^{19}F-containing moiety to a reactive side chain such as cysteine or lysine. The -SH groups on solvent accessible cysteines have been used predominately, and most often 3-bromo-1,1,1-trifluoroacetone (BTFA) or fluorinated phenylacetamide such as 2-bromo-N-(4-(trifluoromethyl) phenyl)acetamide (BTFMA) or N-(4-bromo-3-(trifluoromethyl) phenyl)acetamide (3-BTFMA) are employed in proteins in which any other endogenous cysteines have been replaced by alanines (Edwards et al., 2018; Luchette et al., 2002; Manglik et al., 2015). Lysine side chains can be labeled with n-trifluoroacetylcaproxyloxy succinimide ester (TFCS), a common crosslinker, provided the reaction is carried out below pH 7.5 (Edwards et al., 2018) or may be conjugated to fluorinated indoles and indole-derivatives under mild, nondenaturing reaction conditions (Larda et al., 2015). In addition, enzymatic labeling of the glutamine γ-carboxamide with ^{19}F-2,2,2-trifluoroethylamine hydrochloride (TFEA-HCl) transglutaminases is possible, although, so far, this methodology has only been applied to a few systems (Gimenez et al., 2017; Hattori et al., 2017). Residue-specific ^{19}F labeling is achieved by expression of the protein in defined growth media, supplemented with the ^{19}F-modified amino acid or a suitable precursor, relying on the endogenous aminoacyl-tRNA synthetases to charge their cognate tRNAs with ^{19}F-modified amino acids. In this approach, all residues of that type will be substituted by the ^{19}F-modified amino acid. Unfortunately, this approach does not work for all types of fluorinated amino acids. The efficiency of amino acid-type ^{19}F labeling sometimes can be enhanced using tailored auxotrophic strains. If, however, the fluorinated amino acid is toxic to the cell, specifically evolved orthogonal amber tRNA/tRNA synthetase pairs are needed for labeling. In general, each protein and each substitution with a particular fluorinated amino acid requires careful and systematic testing, and the degree of labeling has to be quantitatively assessed by mass spectrometry: in essence every protein and every type of labeling requires extensive optimization to yield >90% fluorine labeling (Robertson et al., 2020).

At present, there exists a substantial body of literature, documenting the use and power of ^{19}F NMR to gain insight into protein and peptide structure, protein-ligand interactions, protein unfolding, protein aggregation, and protein dynamics. Several reviews have covered the field over the last decades (Danielson and Falke, 1996; Gerig, 1994; Kitevski-LeBlanc and Prosser, 2012; Marsh and Suzuki, 2014). Therefore, here I will focus on the most recent studies and provide an outlook of where I believe fluorine NMR methodology can contribute in unique ways in the future.

Binding and Interactions

^{19}F NMR has been used widely for ligand screening of protein target binding. In such studies, the fluorine tag can be placed on either of the two partners or both. Dalvit and Vulpetti have exploited ^{19}F NMR for dynamic ligand-based and fragment-based screening in drug discovery as well as for functional assessment of enzymatic activities and have reviewed these approaches in detail (Dalvit et al., 2020; Dalvit and Vulpetti, 2019). Such ligand screening approaches are expected to be adopted widely in the pharmaceutical industry and will permit fast and efficient analysis of potential drug candidates. Examples of this type are studies on non-nucleoside inhibitor binding to HIV-1 reverse transcriptase that revealed a highly plastic apo enzyme which rigidified upon inhibitor binding (Sharaf et al., 2016; Sharaf et al., 2017). In this context, ^{19}F lineshape analysis in one-dimensional experiments permits simultaneous determination of thermodynamic equilibrium binding constants (K_d) as well as association (k_{on}) and dissociation (k_{off}) rates (Stadmiller et al., 2020).

NMR studies on membrane proteins, in particular GPCRs, have exploited ^{19}F-labels to investigate conformational changes upon agonist/antagonist binding in this important class of receptors, with the goal to correlate specific receptor conformations to the pharmacological outcome of ligand binding. Indeed, studies of this kind possess an appealing potential for drug screening and drug discovery (see recent reviews by Wang and others (Picard and Prosser, 2021; Wang et al., 2021b)). In most cases, ^{19}F labels are conjugated to naturally present cysteines near the cytoplastic face of the receptor (Frei et al., 2020) or on strategically introduced Cys residues at positions sensitive to signal transduction. Using ^{19}F NMR, it has been possible to identify inactive intermediates and partial- or full-agonist-driven activated states for the adenosine A2A receptor (Huang et al., 2021; Wang et al., 2021a). Such studies inform about immensely important physiological processes, and, given that large numbers of drugs are being designed for pharmacological interventions in GPCR-mediated signaling, fluorine NMR in this context has enormous potential.

The observation of different functional states of biomacromolecules, as well their characterization, is possible by fluorine NMR. For example, transitions between a number of structured active states of the enzyme fluoroacetate dehalogenase throughout the catalytic cycle were carefully assessed by the Prosser group (Di Pietrantonio et al., 2019), a very active, creative and productive group contributing to the ^{19}F NMR field.

As a tool for overall structural characterization of proteins or protein complexes, ^{19}F NMR paramagnetic relaxation enhancement experiments (PREs) can be used to extract quantitative distance information for selectively ^{19}F-labelled, paramagnetically tagged proteins. This was demonstrated as a proof-of-concept by my group (Matei and Gronenborn, 2016), and Konrat and co-workers used this methodology to follow the conformational transition of Myc from a disordered state to the ordered Myc-Max heterodimer state (Somlyay et al., 2020). The Boudker group has also used ^{19}F PREs to monitor domain movement in a glutamate transporter homolog (Huang et al., 2020).

In an interesting application, the ^{19}F-^{13}C TROSY effect for specifically labeled aromatic ring systems was exploited since considerable narrower resonances are observed for ^{13}C nuclei bound to fluorine in comparison to those attached to ^{1}H. Remarkably sharp lines were obtained for a 3F-C_{13} tyrosine-labeled single-ring of the 20S proteasome core particle, a 180-kDa large system, as well as 5-fluorouracil-substituted DNA (Boeszoermenyi et al., 2019). Unfortunately, a tool set of aromatic amino acids tailored to this application is not readily available, but hopefully the unique aspects of ^{19}F-^{13}C TROSY for studying proteins and nucleic acids in complex environments will inspire their further development.

Protein Folding

In the protein folding field, ^{19}F NMR has been relevant for 30 years (Bann et al., 2002; Ropson and Frieden, 1992). A recent study from the Frieden group investigated the conformational equilibrium in ligand-free thrombin, revealing a plastic protein ensemble with ligands binding to the dynamic open form of the enzyme by conformational selection (Ruben et al., 2020). Using real-time ^{19}F NMR on trifluoro-Met35 labeled Aβ_{1-40}, multiple oligomeric species were detected in the initial phase of fiber formation (Suzuki et al., 2013). Likewise, the multistep urea dissociation−unfolding−aggregation equilibrium of the amyloidogenic protein transthyretin has been followed by real-time ^{19}F-NMR. In this study, 6F-Trp-labeled transthyretin was shown to aggregate more rapidly than 5F-Trp- or non-labelled transthyretin, and 5F-Trp-labelled transthyretin was kinetically more stable than the 6F-Trp labelled protein (Sun et al., 2017).

With ever more studies targeting intrinsically disordered (ID) and low complexity regions in proteins, ^{19}F NMR is gaining increasing momentum for investigating such flexible and highly dynamic systems. Early on, the fibrillization of α-synuclein (Syn) was monitored by ^{19}F NMR (Li et al., 2009) as well as its disorder-order transition from aqueous solution to a partial helical conformation in membrane-like environments (Wang et al., 2010). More recently, using a S118C-BTFA fluorinated modified version of the structurally disordered transactivation domain of the estrogen receptor, a metastable long-range contact between residues 33 and 118 was observed in the disordered protein, providing an intriguing structural explanation for linking the oncogenic Ser118 phosphorylation to coactivator binding inhibition (Peng et al., 2019).

Dynamics Studies

In principle, quantitative estimates about molecular motions can be obtained from NMR relaxation parameters. While the interpretation of amide NH relaxation rates has been highly successful for characterizing protein motions (Ishima and Torchia, 2000; Kay, 1998; Mittermaier and Kay, 2006; Salmon and Blackledge, 2015), the use of ^{19}F relaxation rates to assess protein dynamics is fraught with difficulties. As a result, only a handful of ^{19}F-relaxation studies to probe dynamics in biomolecules in solution has been reported in the past 20 years (Hoang and Prosser, 2014; Luck et al., 1996; Peng, 2001; Shi et al., 2011). Recently, we investigated 4F-, 5F-, 6F-, 7F-L-Trp, both, as the free amino acids and when incorporated into the human cyclophilin A protein. Distinct longitudinal and transverse relaxation rates were observed for fluorine atoms at different positions in the indole ring. Comparing experimentally measured and calculated relaxation rates suggests that ^{19}F relaxation for each position in Trp is mainly determined by the ^{19}F CSA and ^{19}F-^{1}H dipolar interactions within the indole ring and only moderately affected by protein motions (Lu et al., 2019a).

Solid State MAS NMR Studies

Exploiting the fluorine nucleus in solid-state NMR has a long history (Griffin et al., 1972). For large biomolecular systems, MAS NMR has distinct advantages over solution methods since it is not limited by the size of the object under study. Therefore, large and complex macromolecular assemblies can be successfully investigated. Furthermore, distance and orientation information can be extracted from dipolar couplings and CSA tensors. Early on, the interaction of fluorine labeled short peptides with membranes was studied, and this work took advantage of the ease with which fluorinated building blocks can be incorporated into chemically synthesized peptides using solid state automated syntheses (Ulrich, 2005). A recent review on this subject is available (Grage et al., 2020). MAS NMR protein studies that have taken advantage of the ^{19}F nucleus include the elucidation of the cholesterol-binding site on the influenza M2 protein in phospholipid bilayers employing a fluorinated cholesterol molecule (Elkins et al., 2017) and inter-fluorine distance measurements in large tubular assemblies of the HIV-1 capsid protein (Wang et al., 2018). Importantly, MAS frequencies of 40–60 kHz were sufficient to obtain adequately narrow lines, obviating the need for decoupling. Furthermore, ^{19}F DNP-enhanced MAS NMR spectroscopy holds very exciting potential for biomolecular applications: significant signal enhancements were observed in ^{19}F DNP spectra at MAS frequencies of 20-25 kHz, and these allowed acquisition of ^{19}F-based DNP HETCOR spectra, which are impossible under conventional experimental conditions (Lu et al., 2019b). I am, therefore, confident that MAS fluorine bioNMR will have far-reaching applications for a wide range of large biological systems.

Novel applications

Reversible clustering

An interesting application of fluorine NMR concerns reversible cluster formation in concentrated mixtures of proteins or nucleic acids, such as that governing the mechanism of liquid−liquid phase separation (LLPS). Such systems can be investigated by dark-state exchange saturation transfer (DEST) experiments. ^{19}F DEST was employed to quantify individual types of protein clusters in highly concentrated mixtures of two differently fluorinated antibodies and revealed that these monoclonal antibodies are involved in separate homogeneous self-associated clusters (Edwards et al., 2019). Although no current reports have emerged that employ ^{19}F NMR to study LLPS of proteins and nucleic acids, it is clearly a very powerful methodology to interrogate this phenomenon. Proteins that can undergo LLPS often contain low-complexity disordered regions and remain dynamic in solution, thereby positioning NMR spectroscopy as a uniquely suited technique for characterization. Fluorine NMR is certainly anticipated to have a large role to play in this arena.

In cell NMR

Most NMR approaches for investigating structure, dynamics and interactions of biomolecules use a divide-and-conquer approach in which the influences of complex biological environments are ignored. This shortcoming spurred the development and rise of in-cell NMR spectroscopy (Freedberg and Selenko, 2014; Li et al., 2017; Luchinat and Banci, 2017; Siegal and Selenko, 2019) aimed at interrogating the behavior of biomolecules under physiological *in vivo* conditions. Using traditional bioNMR methods, in-cell experiments are challenging in multiple ways. A major difficulty arises from the large number of H, C, and N atoms found in cellular components, macromolecules and small metabolites alike. As a result, identifying signals from a molecule of choice among the vast number of background signals from the environment is very complex. Furthermore, the low natural abundance of ^{13}C and ^{15}N isotopes necessitates extensive isotopic labeling for in-depth characterization, and, for large systems or molecules that interact with sizable cellular partners, the short coherence lifetime makes scalar-based magnetization transfers that are exploited in multidimensional NMR spectra problematic or impossible. Therefore, ^{19}F NMR seemed to be a logical choice for in-cell use. Initially used for the small protein GB1 and its variants in *E.coli* cells (Ye et al., 2013; Ye et al., 2019), it recently has been used in *X. laevis* oocytes to evaluate the monomer-dimer equilibrium of the A34F GB1 dimer (Speer et al., 2021). The dynamics of post-translational modification, in particular phosphorylation and dephosphorylation of α-synuclein, was also studied in *X. laevis* oocytes (Selenko et al., 2008), as well as in neuronal SK-N-SH cells (Zheng et al., 2019). The authors showed that α-Syn is more stable in SK-N-SH cells than in oocytes and that S129 phosphorylation accelerates protein degradation. Using a particular variant, Y125E/Y133E α-synuclein, in which dephosphorylation is slowed down, dephosphorylation and degradation of a-synuclein in real-time could be followed in living cells by fluorine NMR (Zheng et al., 2019). Likewise, real-time monitoring of endogenous thiols in mammalian HeLa cells has been possible, using a tailored fluorinated small molecule reagent, 2,3,5,6-tetrafluroro-4-(phenylsulfonyl)pyridine and derivatives thereof (Chai et al., 2020). For illustration purposes, spectra of 3-F-Tyr labeled α-synuclein in cell lysate and mammalian cells recorded in my laboratory are provided in Figure 3.

An ingenious method for site-specifically labeling endogenous proteins with fluorine, termed ligand-directed tosyl (LDT) chemistry, was developed by the Hamachi group. It entails high-affinity binding of a ligand to a target protein and driving the labeling reaction of a nearby amino acid with a reactive group through the proximity effect. The main advantage of this approach is that labeling can be accomplished near an active site of an enzyme without compromising protein function. They showed that it was possible to label carbonic anhydrase with a bis(trifluoromethyl)benzene derivative in red blood cells and that the fluorinated carbonic anhydrase in the cell can act as a biosensor for detecting ligand binding to carbonic anhydrase *in cellulo*, using the ^{19}F NMR signal as a readout (Tsukiji et al., 2009). Interestingly, the exchange rate for the carbonic anhydrase inhibitor benzenesulfonamide in red blood cells was 1.6-fold faster than *in vitro*, suggesting that ligand release is enhanced in the cellular environment (Takaoka et al., 2013).

My own laboratory has recently used fluorine NMR in mammalian cells to provide proof of principle that^{19}F chemical shifts are sensitive to quinary interactions in the cell and that ^{19}F-PREs can be measured and used to assess protein conformations in a native environment (data not shown).

At present, most in-cell fluorine NMR investigates the behavior of proteins and only very few studies are addressing nucleic acids. Only one group has reported ^{19}F NMR studies on nucleic acids in a cellular environment. Specifically, using phosphoramidite chemistry, a 3,5-

bis(trifluoromethyl)benzene moiety was attached to the 5′ terminus of an RNA oligonucleotide and ^{19}F-labeled telomeric RNA G-quadruplexes were prepared using this RNA. Injecting these quadruplexes into *Xenopus laevis* oocytes permitted their investigation by ^{19}F NMR. The authors provide compelling evidence that in oocytes, telomeric RNA G-quadruplexes preferentially adopt a stacked higher order structure composed of two G-quadruplex subunits, rather than a single-unit species (Bao et al., 2017). In contrast, in HeLa cells, hybrid-1, hybrid-2 and two-tetrad antiparallel G-quadruplex structures were observed (Bao et al., 2019). The group has also investigated formation of telomeric DNA-RNA hybrid G-quadruplexes (Bao and Xu, 2020).

Outlook

Currently, there remains a disconnect between *in vitro* and *in vivo* studies by NMR. It will become more and more important to provide and compare quantitative information on the systems under investigation, both in isolation and in the complex environment of a cell. NMR, both solution and MAS solid state NMR, can help to connect atomic-level observations of isolated and in-cell molecules. Taking a multi-method integrated approach will allow the field to develop an increasingly multi-perspectival and more accurate view of the molecules when they perform their function. It is intriguing that some tags that are used for cellular fluorescence microscopy contain fluorine atoms, and it will be instructive to connect the findings from imaging approaches to answers obtained by fluorine NMR. Many questions remain open: Are there any differences in structure or dynamics depending on the environment? Are these functionally important? What confers specificity to components within distinct complex mixtures or cellular locations? How does assembly and disassembly occur and what are the spatial and temporal boundary conditions? Perhaps the unique properties of fluorine, both with respect to its unusual chemical nature and attractive NMR behavior, will help answer some of these inquires.

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Notes

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Table 1. Properties of biologically important NMR nuclei

Nuclei	Natural abundance (%)	Spin (I)	Gyromagnetic ratio, γ (rad sec^{-1} x gauss^{-1})	Sensitivity (% versus ^{1}H)	Van der Waals radius (Å)
^{1}H	99.98	1/2	26,753	100	1.2
^{2}H	0.016	1	4,106	0.965	1.1999
^{13}C	1.108	1/2	6,728	1.59	1.7
^{15}N	0.37	1/2	-2,712	0.104	1.55
^{17}O	0.037	5/2	-3,628	0.00111	1.52
^{19}F	100	1/2	25,179	83.3	1.47
^{31}P	100	1/2	10,841	6.63	1.8

Figure Captions

Figure 1. Superposition of 1D ^{19}F spectra of 4F-Trp (gray), 5F- (magenta), 6F-Trp (green) and 7F-Trp (blue), and 4F-, 5F-, 6F-, and 7F-Trp121 labeled CypA. Chemical shift differences between the free amino acid and the amino acid when incorporated into the CypA protein are indicated by the dashed arrows. Also depicted are the chemical structure of the Trp amino acid with numbering in the indole ring (top) and a ribbon representation of the CypA structure with an expansion of the active site (right), illustrating the superposition of the differently fluorinated

Trp121 side chains. The color coding of the fluorine positions in the structures is identical to that in the spectra.

Figure 2. The three major methods to prepare ^{19}F-modified proteins. (Left) covalent conjugation of ^{19}F-containing moieties to the protein. (Middle) Biosynthetic amino acid type-specific incorporation of ^{19}F-modified amino acids. (Right) Site-specific incorporation of ^{19}F-modified amino acids using recombinantly expressed orthogonal amber tRNA/tRNA synthetase pairs.

Figure 3. Superposition of 1D ^{19}F spectra of 3F-Tyr labeled α-synuclein in buffer (black dotted line), in cell extract (grey line) and in A2780 cells (black line). Assignments are indicated by residue name and number. A schematic ribbon representation of a single α-synuclein chain with the four Tyr residues shown in stick representation is depicted above the spectra.

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