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Letter

Site-Specific Incorporation of 7-Fluoro-L-tryptophan into Proteins by Genetic Encoding to Monitor Ligand Binding by ^{19}F NMR Spectroscopy

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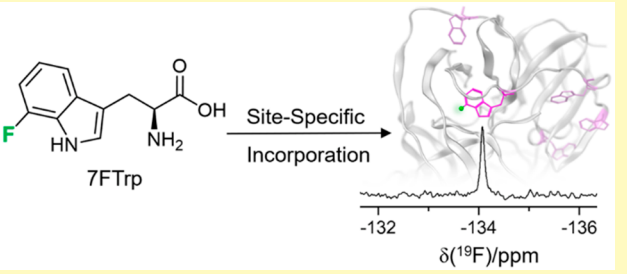
ABSTRACT: A mutant aminoacyl-tRNA synthetase identified by a library selection system affords site-specific incorporation of 7-fluoro-L-tryptophan in response to an amber stop codon. The enzyme allows the production of proteins with a single hydrogen atom replaced by a fluorine atom as a sensitive nuclear magnetic resonance (NMR) probe. The substitution of a single hydrogen atom by another element that is as closely similar in size and hydrophobicity as possible minimizes possible perturbations in the structure, stability, and solubility of the protein. The fluorine atom enables site-selective monitoring of the protein response to ligand binding by ^{19}F NMR spectroscopy, as demonstrated with the Zika virus NS2B-NS3 protease.



KEYWORDS: *fluoro-tryptophan, ^{19}F NMR spectroscopy, ligand binding, genetic encoding, noncanonical amino acids, pyrrolysyl-tRNA synthetase*

^{19}F NMR spectroscopy of proteins labeled with fluorine atoms is becoming increasingly popular for studying protein structure, conformational changes, and protein−ligand interactions.[1−3] ^{19}F is a stable nucleus with 100% natural abundance that is absent from biological macromolecules, resulting in clean signals in the ^{19}F NMR spectrum. C−F groups feature similar steric and hydrophobic properties as C−H groups, leading to minimal structural perturbation.[4,5] Furthermore, ^{19}F chemical shifts are exquisitely sensitive to changes in the chemical environment, featuring a chemical shift range of more than 300 ppm.[6]

Usually, fluorine probes are installed in proteins either via chemical tags or noncanonical amino acids (ncAA). Chemical modification of a single solvent-exposed cysteine residue with probes containing CF_3-groups has proven to be a powerful tool for monitoring conformational changes in proteins such as G-protein coupled receptors, but depends on the absence of other reactive cysteine residues in the protein.[7,8] Genetic encoding systems, which incorporate ncAAs in response to an amber stop codon, exist for a number of fluorine-containing ncAAs, including trifluoromethyl-L-phenylalanine,[9,10] trifluoromethyl-L-tyrosine,[11] N^{ϵ}-trifluoroacetyl-L-lysine,[12] and *p*-pentafluorosulfanyl-L-phenylalanine.[13] These strategies introduce new and relatively bulky chemical moieties into the protein, which may cause unintended changes in structure and function. In an alternative approach, a natural amino acid can be modified by substituting a single hydrogen atom for a fluorine atom. In many cases, such as fluorotryptophans (FTrp), the wild-type aminoacyl-tRNA synthetases (RS) readily accept the fluorinated amino acids in place of their natural substrates, leading to proteins with uniform substitution of tryptophan by FTrp residues. This strategy has been successfully employed for a long time.[14−17] For proteins containing more than a single tryptophan residue, however, the assignment of the ^{19}F NMR signals of individual FTrp residues is difficult, as scalar ^{1}H−^{19}F couplings are rarely resolved and the ^{19}F chemical shifts may change, when resonance assignment is attempted by mutating individual residues to other aromatic residues such as phenylalanine or tyrosine.[18,19] Although global replacement of tryptophan by fluorinated tryptophan analogues requires auxotrophic *Escherichia coli* strains or special growth media,[19−22] this metabolic labeling method presents the current state of the art, which has been widely applied also with a variety of other fluorinated amino acids.[23−26]

To assign the ^{19}F NMR signals while minimizing structural perturbations, it would be advantageous if a singly fluorinated amino acid could be selectively incorporated in response to an amber stop codon. This is challenging, as the change in size and chemical property resulting from substitution of a single

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hydrogen atom with fluorine is minimal. The van der Waals radius of fluorine (1.47 Å) is only a little larger than that of hydrogen (1.2 Å), and typical C−F bond lengths differ from C−H bond lengths by less than 0.3 Å. Without additional changes in the amino acid,[27] previous attempts of site-specific *in vivo* incorporation of a singly fluorinated ncAA invariably resulted in heterogeneous samples with either canonical amino acids inserted into the target site or the fluorinated ncAA randomly replacing canonical amino acid residues elsewhere in a global substitution.[28−30]

Encouraged by reports of successful genetic encoding of tryptophan analogues with small chemical modifications such as cyanotryptophans,[31] 5-hydroxyl-tryptophan,[32,33] 5-bromo-tryptophan,[33] 7-chlorotryptophan,[34] methyl-tryptophans,[35] and 3-benzothienyl-alanine,[35] we sought an RS enzyme capable of discriminating between fluoro-tryptophan and natural tryptophan. Here, we describe the identification of an RS enzyme that enables *in vivo* production of proteins with single atom substitution by genetic encoding of 7-fluorotryptophan (7FTrp). We demonstrate the site-specific incorporation of 7FTrp into the 25 kDa Zika virus NS2B-NS3 protease (ZiPro) for monitoring ligand binding by ^{19}F NMR spectroscopy.

Using our previously reported library screening approach based on the pyrrolysyl-tRNA synthetase derived from the methanogenic archaeon ISO4-G1 (G1PylRS) and fluorescence-activated cell sorting (FACS),[13,36,37] we identified G1PylRS enzymes that specifically recognize 7FTrp (Figure 1a and c). In contrast, selection for G1PylRS enzymes specific for 4-fluorotryptophan (4FTrp; Figure 1b) carried out in parallel was unsuccessful (Figure 1d).

The selection of 7FTrp-specific RS enzymes was conducted in *E. coli* DH10B cells cotransformed with the selection plasmid pBAD-H6RFP, which harbors the gene of mCherry red fluorescent protein (RFP) preceded by an amber stop codon and an N-terminal His_6 tag (His_6-TAG-RFP), and the plasmid pBK-G1RS containing the G1PylRS library (Leu124, Tyr125, Ala221, and Trp237 fully randomized to the 20 natural amino acids; Asn165 selectively mutated to Gly/Ala/Val/Ser/Asn/Thr/Ile/Asp; Val167 selectively mutated to Gly/Ala/Val/Ser/Cys/Leu/Phe; Tyr204 selectively mutated to Phe/Tyr/Trp).[37] The cells were cultured under positive and negative selection conditions in subsequent screening rounds, with independent selections targeting 7FTrp or 4FTrp carried out in parallel (Figures 1, S1, and S2). The positive growth condition included 2.5 mM of FTrp supplied to LB medium. Cells with high levels of RFP fluorescence in positive selection rounds were collected, and negative selection rounds were performed to select cells with low RFP expression. Three positive and two negative rounds were conducted in an alternating manner, culturing the appropriate cell fraction identified by FACS sorting after each round. The selection for 7FTrp yielded a significant population of cells after the **5P** round, which showed high RFP fluorescence in the presence of 7FTrp (10.1%) as opposed to the absence of 7FTrp (0.2%; Figure 1c). In contrast, the selection process targeting 4FTrp showed no such enrichment (Figure 1d). While this result may reflect the difficulty to distinguish between 4FTrp and natural tryptophan by mutants in our G1PylRS library, it may in part also be caused by the greater toxicity of 4FTrp to *E. coli* growth observed during the selection process, where the **3P** and **5P** rounds produced less RFP with than without 4FTrp. Seven candidates from the 7FTrp selection that produced the highest RFP fluorescence were isolated and sequenced (Table S1).

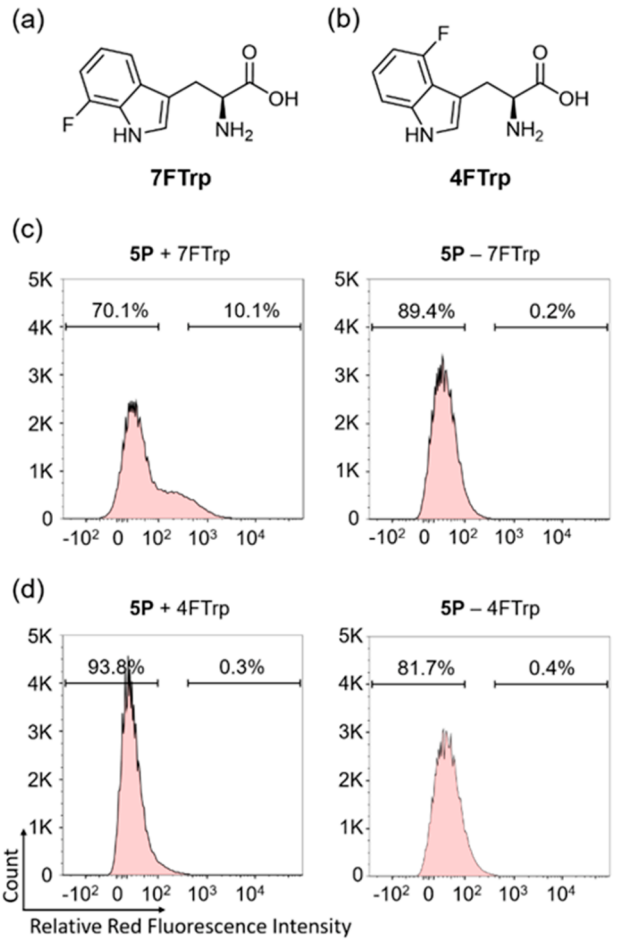

Figure 1. Chemical structures of (a) 7-fluoro-L-tryptophan (7FTrp) and (b) 4-fluoro-L-tryptophan (4FTrp). (c) Fluorescence-activated cell sorting (FACS) analysis of the fifth selection round to identify active G1PylRS enzymes for 7FTrp. Histograms show the number of cells (count) with respect to their red fluorescence intensity. **P** stands for positive selection rounds, where cells were cultured in the presence of FTrp. Identical cell cultures omitting FTrp (−FTrp) serve as controls. Cells were fluorescent when the gene of mCherry red fluorescent protein (RFP) containing an amber stop was expressed successfully. A population of cells with high RFP fluorescence in the presence but not in the absence of 7FTrp indicates the presence of FTrp-specific RS enzymes in the gene pool. (d) Same as (c), but for 4FTrp. The fraction of cells with high RFP fluorescence did not increase in the presence of 4FTrp, i.e., enrichment of active G1PylRS enzymes was observed only in the selection process targeting 7FTrp.

The following experiments used the mutant producing the highest level of fluorescence (referred to as G1(7FTrp)RS). The substrate binding pocket, which carries the mutation set L124G, Y125F, N165G, V167F, Y204W, A221G, and W237Y, was modeled using Rosetta protein structure prediction tools[38] and shows aromatic residues forming strong hydrophobic interactions with 7FTrp (Figure S3).

To create an *in vivo* expression system that incorporates 7FTrp in high yield via amber stop codon suppression, we followed the previously established strategy[36] of cloning the genes of G1(7FTrp)RS and $^{G1Pyl}tRNA_{CUA}$ into a high-copy-number pRSF plasmid (yielding the plasmid pRSF-G1-(7FTrp)RS), while the gene of the target protein with the amber stop codon was cloned into a low-copy-number pCDF plasmid. To test the fidelity of 7FTrp incorporation, RFP with

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an amber stop codon in position 13 (RFP-13TAG) was expressed in culture medium supplemented with 2 mM 7FTrp. Intact protein mass spectrometry of the purified protein revealed protein with a single 7FTrp residue as the most abundant species, but protein containing two 7FTrp residues occurred, too, in about 30% abundance (Figure 2a), illustrating

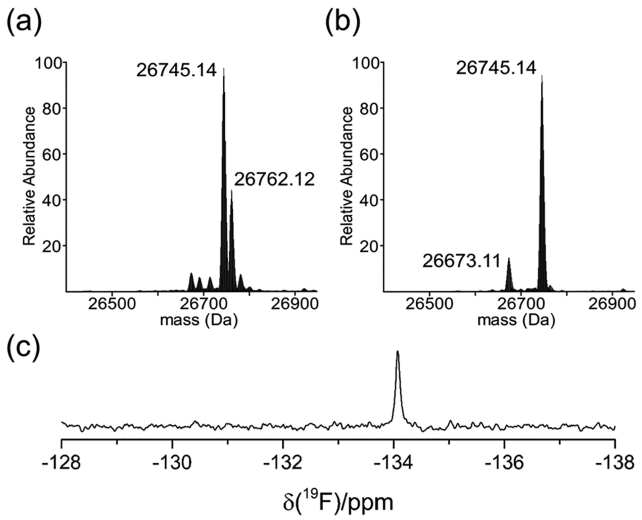

Figure 2. Characterization of protein with single-site incorporation of 7FTrp. (a) Intact protein mass spectrum of RFP-13TAG expressed in a culture medium supplemented with 2 mM 7FTrp. The calculated mass for protein with a single 7FTrp residue in position 13 is 26,745.09 Da. The peak at 26,762.12 Da corresponds to the additional installation of a 7FTrp residue in place of a natural tryptophan residue. (b) Intact protein mass spectrum of RFP13-TAG expressed in a culture medium supplied with 2 mM 7FTrp and 4 mM tryptophan. The mass of the minor peak at 26,673.11 Da corresponds to amber stop codon suppression by glutamyl-tRNA. (c) 1D ^{19}F NMR spectrum of the RFP-13TAG sample in (b), demonstrating the incorporation of a single 7FTrp residue. The spectrum was recorded of a 0.2 mM protein solution in phosphate-buffered saline (PBS) at 25 °C on a 9.4 T NMR spectrometer equipped with a room temperature broadband probe.

the capacity of the wild-type *E. coli* tryptophanyl-tRNA synthetase to incorporate 7FTrp (wild-type RFP contains three tryptophan residues).[39,40] Misincorporation of 7FTrp in place of natural tryptophan was strongly suppressed, however, when the culture medium was supplemented with 4 mM tryptophan in addition to 2 mM 7FTrp, without affecting the fidelity of 7FTrp incorporation at the amber stop codon site, as indicated by intact protein mass spectrometry and ^{19}F NMR spectroscopy (Figure 2b and c). Mass spectrometry analysis indicated that even the low level of amber stop codon suppression by glutamyl-tRNA was more prominent than misincorporation of natural tryptophan (Figure 2b). G1-(7FTrp)RS also proved to discriminate against 4FTrp, yielding very little protein when 4FTrp instead of 7FTrp was provided in the growth medium (Figure S4). These results illustrate the high specificity of G1(7FTrp)RS for 7FTrp.

To demonstrate the clean incorporation of single 7FTrp residues in different sites of a protein and the utility for monitoring ligand binding, we replaced every individual tryptophan residue in ZiPro by 7FTrp, by changing their codons to the amber stop codon. ZiPro is a serine protease composed of the N-terminal domain of NS3 (NS3pro) fused via a Gly_4-Ser-Gly_4 linker to a short segment of NS2B. The protein is an established drug target.[41,42] The wild-type protein contains six tryptophan residues (Trp61*, Trp5, Trp50, Trp69, Trp83, and Trp89, where the residue in NS2B is denoted by an asterisk). Protein expression yields varied depending on the location of the amber stop codon with varying degrees of amber suppression by glutamyl-tRNA which, in all cases, was much more prominent than misincorporation of natural tryptophan (Figures S5, S6, and Table S3). Figure 3 shows the ^{19}F NMR spectra of the six individual samples in the presence and absence of an inhibitor. The ^{19}F NMR spectra of the six ZiPro 7FTrp mutants recorded without the inhibitor show a single ^{19}F resonance between −131 and −135 ppm, except for the mutants of sites 69 and 83, which also show a second minor peak (Figure 3c). A ^{19}F−^{19}F NOESY spectrum recorded of ZiPro with 7FTrp in position 83 displayed an exchange cross-peak between the two ^{19}F NMR signals, revealing the second peak as originating from a minor conformational species of the same protein rather than chemical sample heterogeneity (Figure S7). Addition of 4-nitrophenyl-4-guanidinobenzoate, which is a generic active-site inhibitor of flaviviral proteases (Figure 3b)[43,44] significantly altered the ^{19}F NMR spectra of the ZiPro 7FTrp mutants (Figure 3c), producing a single signal for the mutants carrying 7FTrp at sites 69 or 83, broadening the signal for the mutant in position 50 beyond detection, and causing chemical shift changes at the other sites, except for the mutant with 7FTrp in position 5, which is in a structurally flexible part of the protein as evidenced by the narrowest line width in the NMR spectrum and the absence of electron density in the crystal structure. Among the tryptophan residues, Trp50 is located closest to the catalytic triad, and this residue would therefore be expected to experience a large effect from the binding of the inhibitor. This is reflected in the apparent exchange broadening observed in the presence of the inhibitor, which may arise from μs/ms dynamics in the active site in conjunction with large chemical shift changes preventing access to the fast averaging regime.

E. coli tryptophan synthase is known to convert haloindoles into the corresponding halotryptophans.[16,45] We therefore explored the use of 7-fluoroindole (7FI) instead of the amino acid for the inexpensive production of proteins with 7FTrp. Supplementing the culture medium with 1 mM 7FI proved to incorporate 7FTrp into ZiPro W89TAG with similar fidelity as using a mixture of 7FTrp and tryptophan (Figures S5, S6, and Table S3). Although 7FI in place of 7FTrp reduced the protein yield by about 25%, 7FI is an order of magnitude less expensive than 7FTrp and thus presents an opportunity for significant cost savings.

In conclusion, genetic encoding of 7FTrp enables the production of proteins, where a single hydrogen atom is site-specifically replaced by a fluorine atom, minimizing any perturbation of protein properties. The approach produces highly homogeneous samples, which can be quickly analyzed by simple 1D NMR, requiring only a modest amount of protein. Using a 600 MHz NMR spectrometer equipped with a ^{19}F cryoprobe, we obtained 10-fold increased sensitivity compared to the setup used in Figure 3c, enabling the detection of the ^{19}F NMR signal of a 20 μM protein solution in a few minutes. Importantly, site-specific incorporation of 7FTrp obliterates the need to assign the ^{19}F NMR signal with the help of separate experiments, which can be difficult in the conventional approach, where all tryptophan residues are replaced by fluorinated analogues. Moreover, the system does not depend on auxotrophic *E. coli* strains or special growth

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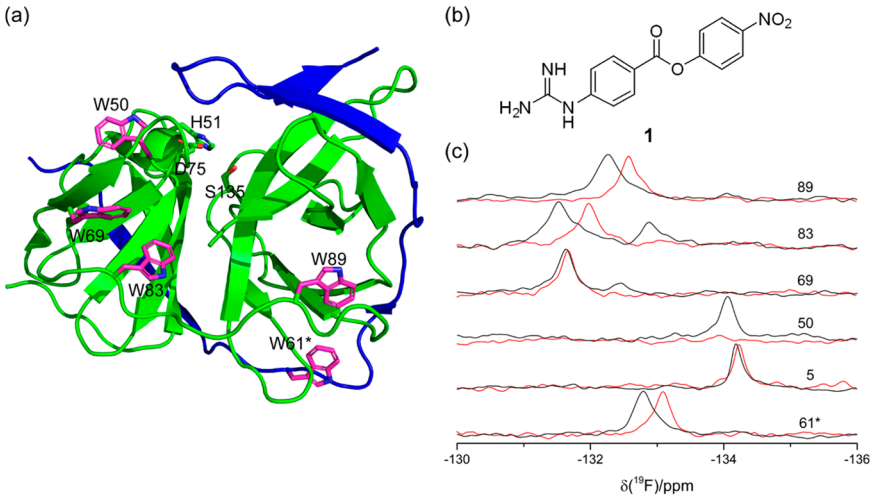

Figure 3. ZiPro with single-point mutations of tryptophan to 7FTrp. (a) Cartoon representation of the crystal structure of ZiPro (PDB ID: 5LC0).[31] NS3pro and NS2B are shown in green and blue, respectively. The side chains of the tryptophan residues are highlighted in magenta (the crystal structure did not report electron density for Trp5). Side chain atoms of the catalytic triad (Ser135, His51, and Asp75) are displayed as sticks. (b) Chemical structure of the broadband protease inhibitor 4-nitrophenyl-4-guanidinobenzoate. (c) 1D ^{19}F NMR spectra of the six ZiPro 7FTrp mutants recorded in the absence (black spectra) and presence of 20 equiv of the inhibitor **1** (red spectra). The spectra were recorded of 0.3−0.7 mM protein solutions in NMR buffer (20 mM MES, pH 6.5, 150 mM NaCl) at 25 °C on a 9.4 T NMR spectrometer equipped with a room temperature broadband probe. The total recording time for each spectrum ranged between 5 and 25 min. The spectra are annotated with the sequence number of the respective mutation sites.

medium. Recent studies highlighted the unique photophysical properties of 7FTrp among tryptophan derivatives,[46,47] further widening the scope of potential applications. The plasmid pRSF-G1(7FTrp)RS has been deposited at Addgene (Watertown, MA) to support wide distribution (Addgene #177310).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.1c02467.

Selection of functional G1PylRS enzymes recognizing 7FTrp; protein expression and purification protocols; model of ncAA binding site of G1(7FTrp)RS; intact protein mass spectrometric analysis of ZiPro with 7FTrp produced with either 7FTrp or 7FI; exchange cross-peaks in 2D ^{19}F−^{19}F NOESY spectrum of ZiPro with 7FTrp in position 83; nucleotide and amino acid sequences (PDF)

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Notes

The authors declare no competing financial interest.

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