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Genetic Encoding of 7-Aza-L-tryptophan: Isoelectronic Substitution of a Single CH-Group in a Protein for a Nitrogen Atom for Site-Selective Isotope Labeling

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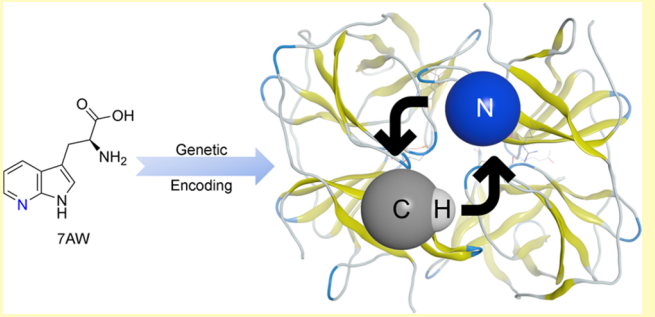
ABSTRACT: Genetic encoding of a noncanonical amino acid (ncAA) in an *in vivo* expression system requires an aminoacyl-tRNA synthetase that specifically recognizes the ncAA, while the ncAA must not be recognized by the canonical protein expression machinery. We succeeded in genetically encoding 7-aza-tryptophan (7AW), which is isoelectronic with tryptophan. The system is fully orthogonal to protein expression in *Escherichia coli*, enabling high-yielding site-selective isotope labeling *in vivo*. 7AW is readily synthesized from serine and 7-aza-indole using a tryptophan synthetase β-subunit (TrpB) mutant, affording easy access to isotope-labeled 7AW. Using labeled 7AW produced from $^{15}N/^{13}C$-labeled serine, we produced 7AW mutants of the 25 kDa Zika virus NS2B-NS3 protease. ^{15}N-HSQC spectra display single cross-peaks at chemical shifts near those observed for the wild-type protein labeled with $^{15}N/^{13}C$-tryptophan, confirming the structural integrity of the protein and yielding straightforward NMR resonance assignments for site-specific probing.



KEYWORDS: *7-azatryptophan, genetic encoding, isoelectronic substitution, NMR spectroscopy, selective isotope labeling*

Selectively isotope-labeled proteins present unique tools for site-specifically interrogating their structure and changes in response to ligand binding. With the advent of algorithms that successfully predict three-dimensional (3D) protein structures from the amino acid sequence with high confidence,[1,2] installing sensitive probes at strategically chosen sites has become important for efficient experimental analysis. Isotope labeling by amino acid type presents excellent probes for analysis by nuclear magnetic resonance (NMR) spectroscopy and minimizes any structural perturbation, but it remains difficult to distinguish the signals in a sequence-specific manner. The commonly used approach of resonance assignment by uniformly isotope-labeled samples and multidimensional spectra poses stringent requirements on sample quality, protein concentration, and molecular weight to achieve the necessary resolution and sensitivity. In contrast, 2D correlation spectra of selectively labeled samples can be obtained much more easily, but the assignment of 2D cross-peaks generally is by site-directed mutagenesis of individual amino acid residues and is easily compromised if mutation of buried residues changes the chemical shifts of the remaining labeled residues.

In an ideal scenario, unambiguous assignments are afforded by a sample where a single amino acid is labeled with stable isotopes. This has been achieved by *in vitro* protein synthesis using separately loaded suppressor tRNA, but the process is laborious and limited by the amount of loaded tRNA.[3−9] Much larger protein quantities with single-residue labeling can be made *in vivo* using photocaged amino acids.[10,11] If installed at a site that is buried in the core of the target protein, however, a photocaging group can severely perturb the protein fold, and for many amino acids (such as tryptophan), genetic encoding with a photocaging group would be difficult. The present work shows that site-specific isotope labeling of tryptophan sites can readily be achieved *in vivo* by using 7-azatryptophan, which is an isoelectronic analogue, readily labeled with isotopes, and suitable for genetic encoding despite its close chemical similarity with tryptophan. The pK_a value of 7-azaindole is 4.59 at 20 °C,[12] indicating that the nitrogen in the six-membered ring is not protonated at neutral pH.

Genetic encoding *in vivo* requires mutant aminoacyl-tRNA synthetases (RS) capable of loading suppressor tRNA with a noncanonical amino acid (ncAA). In order to achieve orthogonality with the host system of protein synthesis, the RS enzyme must not recognize any of the canonical amino

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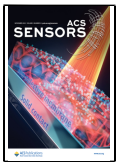


acids, which is possible only if the chemical structure of the ncAA differs from the 20 canonical amino acids. A large variety of ncAA has been genetically encoded in this way.[13−16] RS enzymes display remarkable selectivity. For example, we recently showed that engineered RS enzymes can sense the subtle difference between 7-fluorotryptophan and tryptophan.[17] In the following, we show that 7-azatryptophan (7AW; Figure 1a) can also be genetically encoded, leading

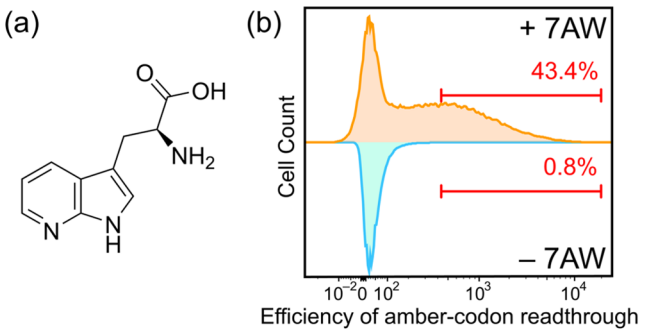
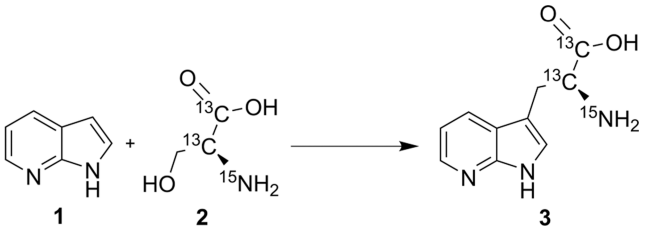


Figure 1. RS enzymes specific for 7-azatryptophan. (a) Chemical structure of 7-azatryptophan (7AW). (b) Histogram of the fifth FACS selection round to identify G1PylRS enzymes active for 7AW. The horizontal axis shows the level of red fluorescence observed by expression of the mCherry red fluorescent protein (RFP) gene, preceded by an amber stop. The vertical axis corresponds to the cell count. The difference in the RFP fluorescence intensity of cells grown with (orange) and without 7AW (negative of cell count; cyan) serves as an indicator of the presence of 7AW-specific RS enzymes in the gene pool.

to proteins with a single CH-group substituted for a nitrogen atom. This minimal change in chemical structure also minimizes any structural perturbation of the protein when tryptophan is mutated to 7AW. We demonstrate the structural conservation by the conservation of the backbone amide chemical shifts in ^{15}N-HSQC spectra of the Zika virus NS2B-NS3 protease (ZiPro), where each of the six tryptophan residues, five of which are buried, was one by one substituted by 7AW labeled with ^{15}N and ^{13}C in the amino acid backbone.

Isotope-labeled 7AW was produced as shown in Scheme 1 from commercially available ^{15}N/^{13}C-labeled serine (Martek)

Scheme 1. Enzymatic Synthesis of Isotope-Labeled 7AW (3) from 7-Azaindole (1) and Serine (2) that was ^{13}C-Labeled in Positions 1 and 2



and unlabeled 7-azaindole (Ambeed), using the *Tm*9D8* mutant of the tryptophan synthase β-subunit (TrpB) from *Thermotoga maritima*, which had been engineered for the conversion of indoles with larger substituents in the 7 position.[18] We expressed the enzyme with an N-terminal His_6 tag in *Escherichia coli* BL21(DE3) with the gene cloned into the multiple cloning site of pET-21(+) (Twist Bioscience). Using 20 μM purified *Tm*9D8* TrpB as the catalyst, 7AW was produced *in vitro* at 55 °C in 72 h from 61 mM ^{15}N/^{13}C-labeled serine and 55 mM 7-azaindole. The conversion of 7-azaindole to 7AW was quantitative based on ^{1}H NMR analysis (Figure S1).

The selection of the requisite RS enzyme succeeded in five rounds of positive and negative selection from a previously published library of RS enzymes derived from the pyrrolysyl-tRNA synthetase of the methanogenic archaeon ISO4-G1 (G1PylRS).[19] The selection used fluorescence-activated cell sorting (FACS) of *E. coli* DH10B cells cotransformed with the G1PylRS mutants on the library plasmid pBK-G1RS and the selection plasmid pBAD-H6RFP, which encodes mCherry red fluorescent protein (RFP) preceded by a His_6-tag and an amber stop codon. The library varied the residues 124, 125, 221, and 237 in full and the positions 165, 167, and 204 in part as described previously.[19,20] Significant enrichment of active G1PylRS enzymes was obtained after the fifth round of selection (Figures 1b, S2, and S3). Candidates yielding the highest level of fluorescence in the presence of 7AW were individually characterized and sequenced (Table S1). The G1PylRS selected contained the mutations L124M, Y125M, N165A, V167G, A221G, and W237H. The gene was subcloned into the high-copy number plasmid pRSF together with the gene of the suppressor tRNA. To confirm the selectivity of the selected G1PylRS mutant for 7AW, a sample of the NT* solubility tag derived from the N-terminal domain of spider silk protein[21,22] was produced at its C-terminal end, which contained a TEV cleavage site followed by aspartic acid and 7AW, so that cleavage with TEV protease yielded a tripeptide with the sequence GD(7AW). Following cleavage, the tripeptide was separated from NT* and TEV protease by Ni-NTA resin purification. The 1D ^{1}H NMR spectrum of the tripeptide was characteristic of 7AW and showed no evidence of natural tryptophan (Figure S4).

7AW mutants of ZiPro were produced *in vivo* in *E. coli* B95.ΔA cells,[23] using LB medium with the isotope-labeled 7AW amino acid provided in 1 mM concentration. The construct contained a Gly_4−Ser−Gly_4 linker connecting the C-terminus of NS2B with the N-terminus of NS3 and a C-terminal His_6-tag for purification using a His GraviTrap column (Cytiva)[20] and the gene was on a pCDF vector with spectinomycin resistance as described previously.[24] 100 mL cultures produced in parallel yielded between 35 and 170 μM samples of purified ZiPro in 0.5 mL NMR buffer (20 mM MES, pH 6.5, 150 mM NaCl). Mass spectrometry was consistent with the incorporation of single 7AW residues (Figure S5).

Figure 2 shows the ^{15}N-HSQC spectra recorded of the six ZiPro samples harboring isotope-labeled 7AW at the six individual sites containing tryptophan in the wild-type protease. As expected, each spectrum shows a single cross-peak. Plotting at the noise level shows no evidence of misincorporation in response to tryptophan codons (Figure S6). The chemical shifts of the 7AW cross-peaks are close to the chemical shifts of the wild-type protein produced with ^{15}N/^{13}C-labeled tryptophan, demonstrating minimal structural perturbation by the introduction of single 7AW residues. The largest difference in chemical shift is observed for position 5, for which the tall cross-peak and the absence of electron density in the crystal structure[25] indicate high flexibility and disorder. The ZiPro construct has a molecular weight of 25 kDa, and 3D NMR experiments of this protein are possible,

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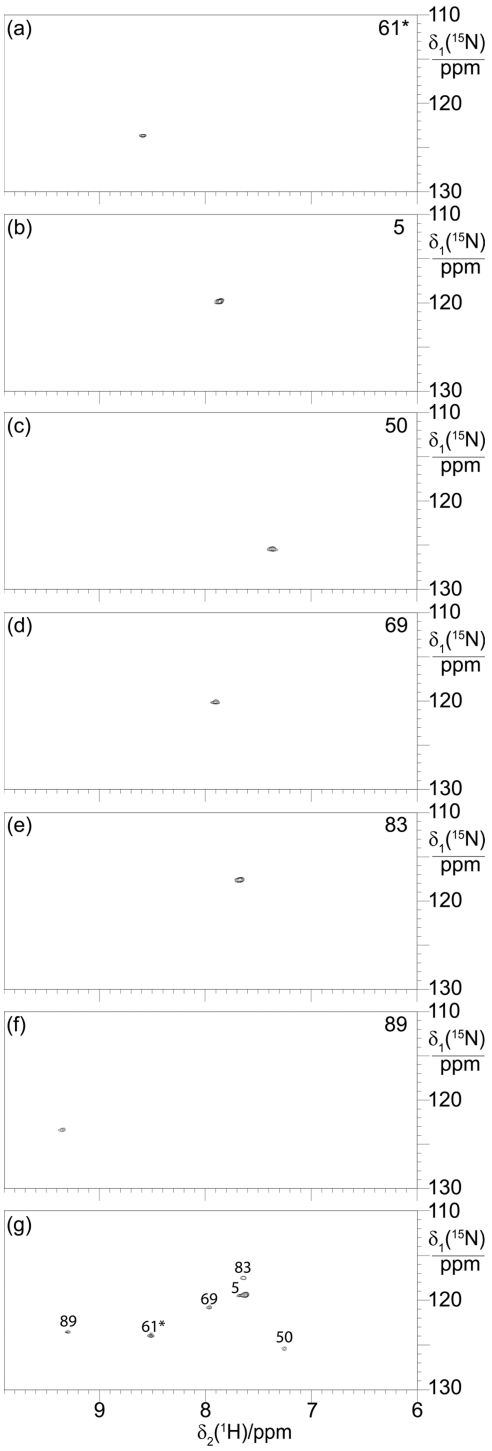

Figure 2. ^{15}N-HSQC spectra of the Zika virus NS2B-NS3 protease mutants with 7-azatryptophan replacing single tryptophan residues. The spectra were recorded on an 800 MHz Bruker AVIII system equipped with a TCI cryoprobe, using total recording times between 2 and 12 h per spectrum. Samples were in NMR buffer (20 mM MES, pH 6.5, 150 mM NaCl) and measured at 25 °C. The spectra are annotated with the sequence number of the mutation sites. (a) ZiPro Trp61*_7AW, where the star indicates the location of the residue in NS2B. The protein concentration was 44 μM. (b) ZiPro Trp5_7AW, 170 μM. (c) ZiPro Trp50_7AW, 140 μM. (d) ZiPro Trp69_7AW, 125 μM. (e) ZiPro Trp83_7AW, 35 μM. (f) ZiPro Trp89_7AW, 75 μM. (g) Reference spectrum recorded for wild-type ZiPro selectively labeled with ^{15}N/^{13}C-labeled tryptophan. Figure S8 shows a superimposition of the spectra.

but resonance assignments of the uniformly labeled protein were laborious and, even for the protein backbone, less than 80% complete due to signal overlap and a wide range of signal intensities.[24] The unique value of ^{15}N-HSQC spectra as a tool for site-specific sensing lies in the extraordinary sensitivity of amide chemical shifts toward changes in the chemical environment. ^{13}C-HSQC spectra of the samples produced in the present work were obscured by buffer signals, buffer impurities, and the water resonance (data not shown).

In conclusion, the selective substitution of single tryptophan residues by 7AW provides a straightforward way of obtaining site-specific resonance assignments. The preparation of isotope-labeled azatryptophan is facile, and the capacity to install 7AW residues *in vivo* also opens the door to affordable site-specific labeling if other residues are to be labeled uniformly, e.g., by perdeuteration as required for NMR analysis of large proteins. Besides applications in protein NMR, azatryptophans also endow proteins with attractive fluorescence properties distinct from tryptophan.[26,27] Site-specifically installed 7AW residues thus carry promise for fluorescent studies of ligand binding and protein folding, as 7AW shows a greater sensitivity to solvent exposure than tryptophan.[28] Among the Zika virus NS2B-NS3 mutants of the present work, 7AW at position 89 stands out for enhanced and red-shifted fluorescence (Figure S7), suggesting lesser solvent accessibility than indicated by different crystal structures.[25,29] We expect that isotope-labeled 7AW will be of great utility not only for protein research in solution but also under conditions where spectral resolution is at a premium, such as dynamic nuclear polarization (DNP)-enhanced solid-state NMR spectroscopy and ENDOR-type EPR measurements with paramagnetic tags in frozen solutions. The ability to install single 7AW residues with the help of conventional rather than Trp auxotrophic *E. coli* strains[26,27,30,31] adds to the attractiveness of the system.

The plasmids pRSF-G1(7AW)RS and pET-21(+)*Tm*9D8* TrpB were deposited at Addgene (Watertown, MA) to support distribution and applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.3c01904.

Protocol for the expression and purification of *Tm*9D8* TrpB; enzymatic synthesis of isotope-labeled 7AW by *Tm*9D8* TrpB monitored by ^{1}H NMR (Figure S1); FACS experiments for the selection of active G1PylRS enzymes specific for 7AW (Figure S2); screening of individual colonies for the activity of G1PylRS for 7AW incorporation (Figure S3); experiment showing the potential of the selected G1PylRS mutant to discriminate between 7AW and tryptophan (Figure S4); intact protein mass spectra of ZiPro mutants with ^{15}N/^{13}C-labeled 7AW (Figure S5); ^{15}N-HSQC spectrum illustrating the selectivity of 7AW incorporation (Figure S6); superimposition of the ^{15}N-HSQC spectra shown in Figure 2 (Figure S7); UV absorption and emission fluorescence spectra of the Zika virus NS2B-NS3 7AW mutants (Figure S8); mutations found in G1PylRS variants selected to recognize 7AW (Table S1); and DNA and the corresponding amino acid sequences of the proteins used in the current study (Table S2) (PDF)

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Notes

The authors declare no competing financial interest.

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