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Site-Specific Incorporation of Fluorotyrosines into Proteins in *Escherichia coli* by Photochemical Disguise[†]

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ABSTRACT: Fluorinated analogues of tyrosine can be used to manipulate the electronic environments of protein active sites. The ability to selectively mutate tyrosine residues to fluorotyrosines is limited, however, and can currently only be achieved through the total synthesis of proteins. As a general solution to this problem, we genetically encoded the unnatural amino acids *o*-nitrobenzyl-2-fluorotyrosine, -3-fluorotyrosine, and -2,6-difluorotyrosine in *Escherichia coli*. These amino acids are disguised from recognition by the endogenous protein biosynthetic machinery, effectively preventing global incorporation of fluorotyrosine into proteins.

The fluorine atom is often considered an isosteric replacement for hydrogen, unlikely to perturb structure, yet with a high electronegativity that enables its application as a biological probe. For example, the pK_a of a tyrosine phenolic proton is approximately 10, but the pK_a of fluorotyrosine residues can range from 5.2 to 9.0 depending on the extent of fluorination (*1*, *2*). Therefore, the acidity of individual amino acid side chains within a protein can be precisely modulated in investigating the participation of a given tyrosine residue in an acid−base catalysis mechanism. The same concept applies to the redox properties of fluorinated tyrosine residues (*3*). In the study of biologically generated tyrosyl radicals, fluorotyrosines have peak reduction potentials that range from 705 to 968 mV, depending upon the extent and position of fluorination. This allows fluorotyrosines to be used as comparison probes for tyrosine, which has a peak potential of 642 mV (*3*).

The main limitation for the precise use of fluorinated amino acids is the process by which they are currently introduced into proteins. Because of the structural similarities to natural amino acids (fluorine has an only 0.15 Å larger van der Waals radius than hydrogen), fluorinated amino acids are often introduced into proteins via global incorporation. For example, *Escherichia coli* cells starved of tyrosine (**1**) can be grown in the presence of **2** or **3** (Scheme 1) to force incorporation of these analogues in place of all tyrosine residues (*4*, *5*). The obvious concern for metabolic labeling is that there is no site control and the sample proteins can contain a multiplicity of fluorinated amino acid substitutions.

Several attempts have been made to address this problem of nonexistent site selectivity. Homogeneous proteins containing fluorotyrosines have been produced by a combination of chemical peptide synthesis and expressed protein ligation (*2*). This is limited to certain locations within a protein, typically at the C-terminus, and can be technically challenging for proteins that are sensitive to denaturation. Unnatural amino acid mutagenesis has been performed in an in vitro protein expression system using amber suppressor tRNAs that are chemically aminoacylated with fluorotyrosines (*6*). This approach is limited by the requirement for a laborious synthesis of the aminoacyl-tRNA and limited protein yields. A more versatile method would be to use in vivo unnatural amino acid mutagenesis based on an orthogonal aminoacyl-tRNA synthetase (aaRS)−tRNA pair enabling protein expression in *E. coli* with fluorotyrosine encoded by the amber stop codon, TAG (*7*). In this paper, we report a general method for this approach by temporarily masking fluorotyrosines from cellular metabolism using a photoremovable protecting group (*8*−*10*).

We first chose to investigate the incorporation of *o*-nitrobenzyl-2-fluorotyrosine (**6**) into proteins using a variant of the *Methanococcus jannaschii* tyrosyl-tRNA synthetase (ONB-YRS) that was previously altered to accept the nonfluorinated amino acid *o*-nitrobenzyltyrosine (**5**) as a substrate (*11*). The synthesis of the caged fluorinated tyrosines **6**−**8** was achieved through complexation of the tyrosines **2**−**4** with Cu^{2+} followed by alkylation with *o*-nitrobenzylbromide [Scheme 1 and Supporting Information (SI)], as previously reported for the conversion of **1** to **5** (*11*). Amino acids **2**−**4** were produced in enantiomerically pure form from the corresponding fluorophenols **9**−**11**, respectively, using the enzyme tyrosine phenol lyase (TPL) (*12*). We assumed that with such a small structural change to the substrate, **6** would be accepted by this enzyme and the amino acid could be inserted into proteins using the previously described machinery. After protein production, the caging group could be removed by light irradiation, revealing the desired fluorinated protein. Unfortunately, repeated attempts at producing protein [using the gene encoding superfolder green fluorescent protein (sfGFP) (*13*) having the permissive V150 codon mutated to TAG] containing **6** with ONB-YRS were unsuccessful. This disruption of substrate specificity from a single fluorine was quite astonishing given that **2** and **3** are both substrates for the endogenous *E. coli* TyrRS.

Thus, we decided to create a new aaRS variant capable of accepting **6** as a substrate. A new library was constructed in which

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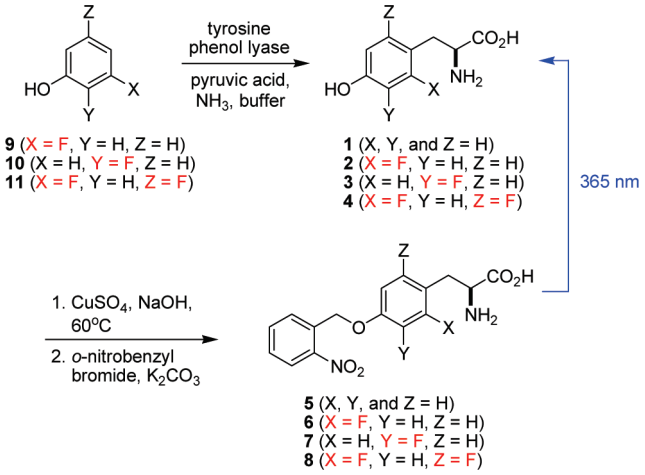
Scheme 1: Structures and Synthesis of Caged Fluorotyrosines



seven residues within the active site of *Mj*YRS (*14*) (Y32, L65, H70, F108, D158, I159, and Ll62) were randomized or selectively diversified, yielding $>10^8$ variants. It should be noted that the original ONB-YRS was derived from a library that did not include randomized residues H70 and I159, resulting in a slightly different starting pool of aaRSs. This new library was then applied in a double-sieve selection experiment, based on suppression of a stop codon in the gene encoding chloramphenicol acetyl transferase (*15*), in which we attempted to isolate a variant that accepts **6** as a substrate (details provided in the SI). After multiple rounds of selection and screening, we isolated two clones, ONB-2FYRS-1 and -2, that grew on agar containing 90 μg/mL chloramphenicol in the presence of **6** but only 20 μg/mL chloramphenicol in the absence of **6** (Figure 1). This suggests that these enzymes are selective for **6** and do not accept endogenous amino acids as substrates. Moreover, examination of the active site sequences of these enzymes revealed that they were homologous, but different from the previously evolved enzyme, suggesting an altered substrate specificity (Table S1 of the SI).

To verify the site-specific insertion of **6** into a protein, we chose to overexpress sfGFP using the variant ONB-2FYRS-1, subcloned into a pSUP plasmid (*16*) for compatibility with our expression system. Expression of the full-length protein was observed only when **6** was included in the growth medium at 1 mM (Figure 1). The protein yield under these conditions was approximately 2 mg/L, which is comparable to the level of protein expression using **5** (*11*). In the absence of **6**, no production of full-length protein is observed on the basis of Coomassie-stained gels and MS. To further verify the correct protein product, we performed an in-gel tryptic digest on the sfGFP protein and subjected the isolated fragments to mass spectrometric sequencing to confirm the site-specific incorporation of the fluorotyrosine (see SI). A comparison of the analysis of sfGFP expressed with ONB-YRS in the presence of **5** and expressed with ONB-2FYRS in the presence of **6** revealed identical peptide masses for both proteins apart from the one fragment containing the mutation which differ by *m*/*z* 18, equal to the mass difference between fluorine and hydrogen. We were curious if the newly developed variants, ONB-2FYRS-1 and -2, of the *M. jannaschii* enzyme would have substrate specificities that are flexible to the other fluorotyrosine analogues, **7** and **8**. Indeed, cells containing either ONB-2FYRS variant supported production of sfGFP containing **7**; however, only ONB-2FYRS-2 produced significant quantities of protein using **8** (Figure 1c,d). The successful incorporation of these amino acids into sfGFP was further verified by mass spectrometry analyses on tryptic fragments of these proteins as described above (see SI).

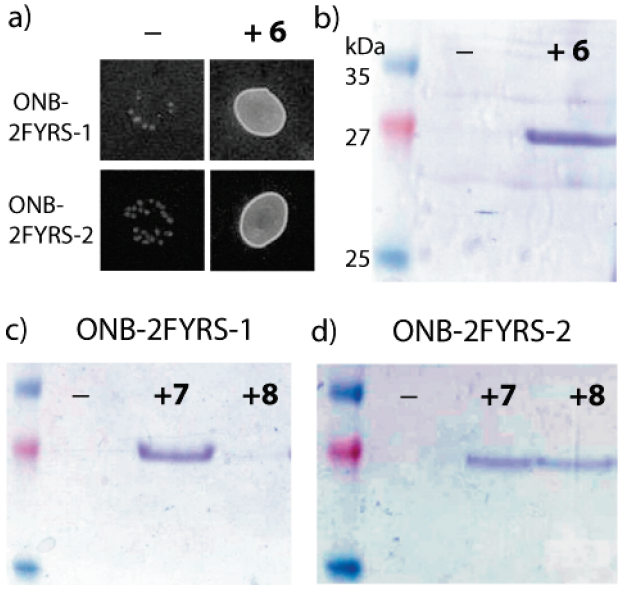


FIGURE 1: Characterization of evolved aaRSs. (a) Growth on glycerol minimal medium with leucine (GMML) agar containing chloramphenicol (90 μg/mL). (b) Expression of sfGFP in the presence and absence of **6**. (c and d) Production of sfGFP containing **7** and **8** using (c) ONB-2FYRS-1 and (d) ONB-2FYRS-2. All protein was isolated by Ni^{2+} affinity chromatography.

We next tested whether site-specific mutations of fluorotyrosines could be used to alter protein function. Previously, global mutagenesis studies have given rise to altered activity, such as pH−rate profiles, but these experiments used proteins containing multiple mutations. One such study used global incorporation of 3-fluorotyrosine into EGFP (*4*, *17*), which contains 11 total tyrosine residues, one of which, Y66, is central to the fluorophore. To conduct a more precise investigation with atom-specific resolution, we expressed and purified sfGFP with only Y66 mutated to **6**−**8**. This protein, unlike any previous studies, contains only a single-residue mutation to a fluorotyrosine. In addition, we produced a control sfGFP, which instead contains **5**, as a surrogate for the wild-type tyrosine residue. All proteins were purified and then "decaged" by irradiation at 365 nm for 30 min, after which we assayed the protein for fluorescence properties. In the case of fluorophore mutation **6** or **8**, containing fluorines in the ortho position, the λ_{em} is blue-shifted (503 or 509 nm, respectively) when compared to the tyrosine control (513 nm) (Figure 2b). Mutations containing the meta-substituted **7** result in a red shift to 515 nm. This demonstrates the ability of a single fluorotyrosine residue to change the electronic properties of the chromophore and ultimately affect the protein function.

Finally, an interesting observation is that the sfGFP bearing **6** is fluorescent at 503 nm, despite the presence of the large *o*-nitrobenzyl group blocking the tyrosine hydroxyl. Fluorescence increases by 343% after removal of the caging group through a brief irradiation at 365 nm (Figure 2c). This increase in fluorescence is most likely due to the liberation of the phenolic hydroxy group which can now delocalize a negative charge throughout the fluorophore ring system, which is not possible when the amino acid is caged. What is not clear is if the sfGFP mutant is fully folded and matured prior to removal of the large

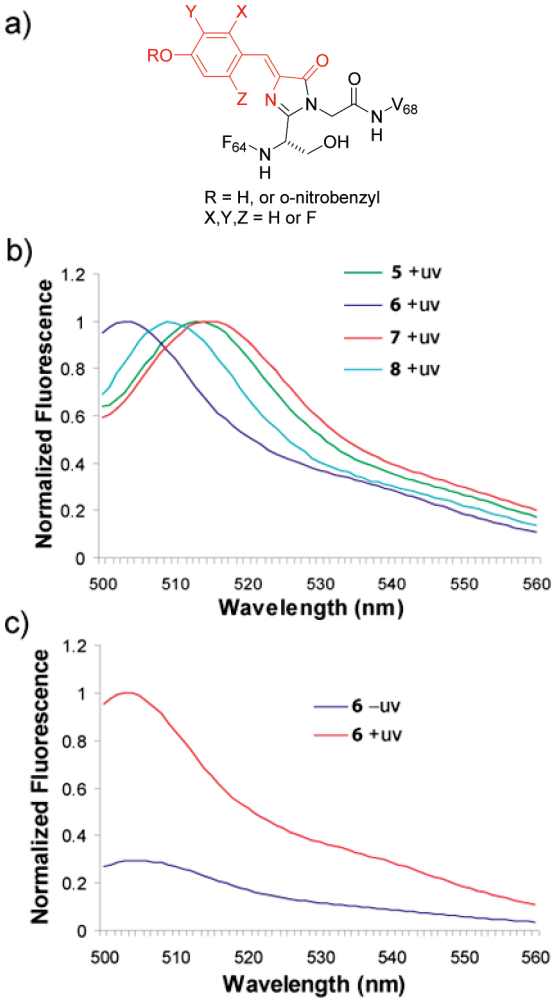


FIGURE 2: Fluorescence analysis of sfGFP containing unnatural amino acids **5**–**8**. (a) Schematic of the GFP active site. (b) Normalized fluorescence of decaged protein containing **5**–**8**. (c) Normalized fluorescence of protein containing **5** before and after irradiation at 365 nm for 30 min showing activation.

ONB caging group. Nevertheless, photoactivation by the removal of a protecting group from the chromophore of the protein could be used as a general method to "switch" fluorescent proteins, and the fact that these molecules are genetically encoded may enable the use of directed evolution to improve the photoswitching properties.

In summary, we developed a general method for the site-specific incorporation of 2-, 3-, and 2,6-fluorotyrosine into proteins in *E. coli*. These near-natural amino acids are temporarily disguised from recognition by the endogenous protein biosynthetic machinery using a light-removable protecting (caging) group. This provides a unique solution to the problem of preventing global incorporation of fluorotyrosine into proteins. We are currently exploring whether this approach might be used for other fluorinated tyrosine analogues (*3*), in which case this methodology will enable the precise investigation of acid–base and redox properties of tyrosine residues.

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SUPPORTING INFORMATION AVAILABLE

Detailed discussion and experimentals, aaRS, and MS analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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