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Synthetase polyspecificity as a tool to modulate protein function

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Abstract

The site-specific incorporation of unnatural amino acids (UAAs) into proteins in bacteria is made possible by the evolution of aminoacyl-tRNA synthetases that selectively recognize and aminoacylate the amino acid of interest. Recently we have discovered that some of the previously evolved aaRSs display a degree of polyspecificity and are capable of recognizing multiple UAAs. Herein we report the polyspecificity of an aaRS evolved to encode a comarin containing amino acid. This polyspecificity was then exploited to introduce several UAAs into the fluorophore of GFP, altering its photophysical properties.

Keywords

Unnatural amino acids; Polyspecificity; Green Fluorescence Protein; Aminoacyl-tRNA Synthetase; Fluorescence modulation

Genetically encoded unnatural amino acids (UAAs) are useful probes of protein structure and functions, and can be used to engineer proteins with novel function.[1] UAAs can be encoded in bacterial, yeast, and mammalian cells in response to a nonsense or frameshift codon by means of an orthogonal aminoacyl-tRNA synthetase (aaRS)/tRNA pair evolved to encode the desired UAA.[2] Using this approach over 70 UAAs with various distinct functions have been incorporated into proteins. The evolution of an aaRS with the desired specificity involves a double sieve selection in which the UAA is first positively selected for incorporation based on chloramphenicol acyltransferase expression, followed by a negative selection based on barnase expression in the absence of the UAA to eliminate aaRSs capable of charging any of the endogenous 20 amino acids. It was recently reported, by us and others, that some aaRSs possess a degree of polyspecificity allowing them to accept multiple UAAs.[3] This polyspecificity arises due to the nature of the selection strategy, as all other UAAs are absent from the selection. This allows evolved aaRSs to be cross-reactive with other UAAs, while still maintaining their orthogonality to endogenous host amino acids and enables the incorporation of multiple amino acids in response to an amber codon using only

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Supplementary Material
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a single aaRS. Herein we describe the characterization of another polyspecific aaRS and its use to alter the fluorescent properties of GFP.

We previously employed a rapid screen to assess the polyspecificity of 15 evolved aaRSs based on GFP fluorescence.[3a] A GFP gene with an amber mutation at Tyr151, GFP_{Y151X} (a surface residue that can be substituted without alteration of the fluorophore) was placed under control of the T7 promoter in a pET101 vector.[4] When co-transformed with a pEVOL plasmid which encodes the desired aaRS/$tRNA_{cua}$ pair, the amber mutation is suppressed in the presence of an unnatural amino acid.[4] To determine if a particular aaRS is capable of recognizing other amino acids, *E. coli* harbouring the two plasmids can be cultured in a 96 well format, with each well containing a different unnatural amino acid. Using this screen the *p*-cyanophenylalanine aaRS (*p*CNFRS)[5] was found to be polyspecific, incorporating over 18 UAAs.[3a] Additionally, the synthetase (CouARS) evolved to incorporate the coumarin (**1**) unnatural amino acid[6] displayed a novel promiscuity for substituted tyrosyl residues (3-iodotyrosine and 3-chlorotyrosine; see Supporting Information). These results prompted us to carry out a secondary screen against structurally similar UAAs (Scheme 1a). Employing the previously described GFP assay, the CouARS was found to efficiently encode 6 unnatural amino acids including 3-iodotyrosine (**2**), 3-chlorotyrosine (**3**), 3-fluorotyrosine (**4**), 3,5-dibromotyrosine (**5**), and 3-vinyltyrosine (**6**) (Scheme 1a). While **2**–**4** have been previously encoded,[7] UAAs **5** and **6** have not been incorporated in bacterial by an orthogonal aaRS/tRNA strategy. No incorporation of UAAs **7**–**10** was observed, potentially due to either their size (**10**) or the nucleophilicity/ionic character of their substituents (**7**–**8**). The polyspecificity of the CouARS may arise from the increased size of the amino acid binding pocket, as key active site residues are mutated to glycine (A67G, H70G, D158G, and L162G). Additionally, the replacement of Y32 with a glutamic acid residue facilitates a greater degree of structural flexibility while still able to engage in hydrogen bonding with the hydroxyl group of tyrosine derivatives.

Unnatural incorporation was confirmed in triplicate for GFP assays, and also by incorporation of the unnatural amino acid into a his-tagged myoglobin mutant containing an F107TAG amber mutation (Myo_{F107X}).[4] Myoglobin was purified using a Ni-NTA resin and analyzed by SDS-PAGE. The calculated mass of the purified mutant protein was confirmed by liquid chromatography/mass spectroscopy (LCMS), and in the presence of any of these amino acids (>1mM), no incorporation of a common twenty amino acid was observed within the detection limits of LCMS (Table 1). While suppression yields are somewhat low (Table 1), they are comparable to the suppression efficiencies with **1** for myoglobin expression (data not shown).

This polyspecificity can be employed to subtly modulate the acidity, electronic nature, and size of a single residue of a protein, by simply including the desired UAA during expression of the amber mutant of interest. This obviates the need for a separate aaRS system for each UAA, and may even preclude the necessity for aaRS selection. As a proof-of-principle experiment we selected GFP, and introduced a TAG codon at Tyr66 (pET-GFP_{Y66X}). This tyrosine residue is integral to the flurorophore, and thus alterations in its structure and electron donating capacity have previously been demonstrated to alter the fluorescence of the protein.[1e, 8] Co-transformation of pEVOL-*p*CNF or pEVOL-CouA with pET-GFP_{Y66X} into BL21(DE3) cells, followed by expression in the presence of 1 mM UAA (induction with 1 mM IPTG, 0.02% arabinose at OD_{600} 0.8; 16h, 30 °C) resulted in production of several previously uncharacterized GFP mutants possessing a UAA within the fluorophore. After purification using a Ni-NTA column and dialysis, the fluorescence spectra of the GFP mutants were measured (Figure 1).

Gratifyingly, the fluorescent properties of GFP can easily be modulated by incorporating different UAAs at position 66 (a fact previously demonstrated).[1e] However, this can now rapidly be achieved using a single aaRS, rather than requiring expression with multiple distinct UAA/aaRS systems. Using the CouARS we were able to incorporate tyrosine derivatives **2**, **3**, and **6** at position 66 in GFP (Scheme 1). The fluorescence spectra of these analogs exhibited a bathochromic shift, with the most substantial shift occurring with the 3-vinyltyrosine **6** (Figure 1). The fluorescence lifetimes and quantum yields for derivatives **2**, **3**, and **6** are similar to GFP with **6** showing the highest quantum yield (0.53) and longest fluorescence lifetime (3.3 ns) (Table 2 and Supporting Information).

Next the *p*CNFRS was used to incorporate a series of *p*-substituted phenylalanine derivatives (**11**–**17**, Scheme 1), resulting in a hypsochromic shift of the GFP fluorescence (Figure 2). The electron donating ability of these functional groups is lower than the natural hydroxyl group of tyrosine accounting for the blue-shifted spectra. This trend is also apparent within the series of derivatives as the most strongly donating azide substituent (**15**) affords the least shifted emission spectrum, followed by the phenyl (**16**) and alloxy (**17**) substituents (Figure 1). In addition, the replacement of the hydroxyl group of tyrosine reduces the fluorescence quantum yields (Table 2). Interestingly, the nitro substituent (**12**) appears to quench fluorescence almost completely ($\phi_f \leq 0.005$).

In summary, we have successfully demonstrated the ability to modulate protein function by the site-specific incorporation of a variety of UAAs using a single aaRS. With this technology it is feasible to both red- and blue-shift the emission spectra of GFP as a means to confer new photophysical properties. Additionally, by exploiting the polyspecificity of the coumarin aaRS, we have also been able to incorporate two previously unencoded UAAs (**5** and **6**). These represent potentially useful probes for the alteration of tyrosine electrochemical properties, and in the case of **6**, can serve as a radical trap or metathesis precursor. Thus, aaRS polyspecificity represents a novel mechanism to rapidly encode a variety of amino acids using a singular expression system allowing for a systematic study of the effect of UAA incorporation on protein structure and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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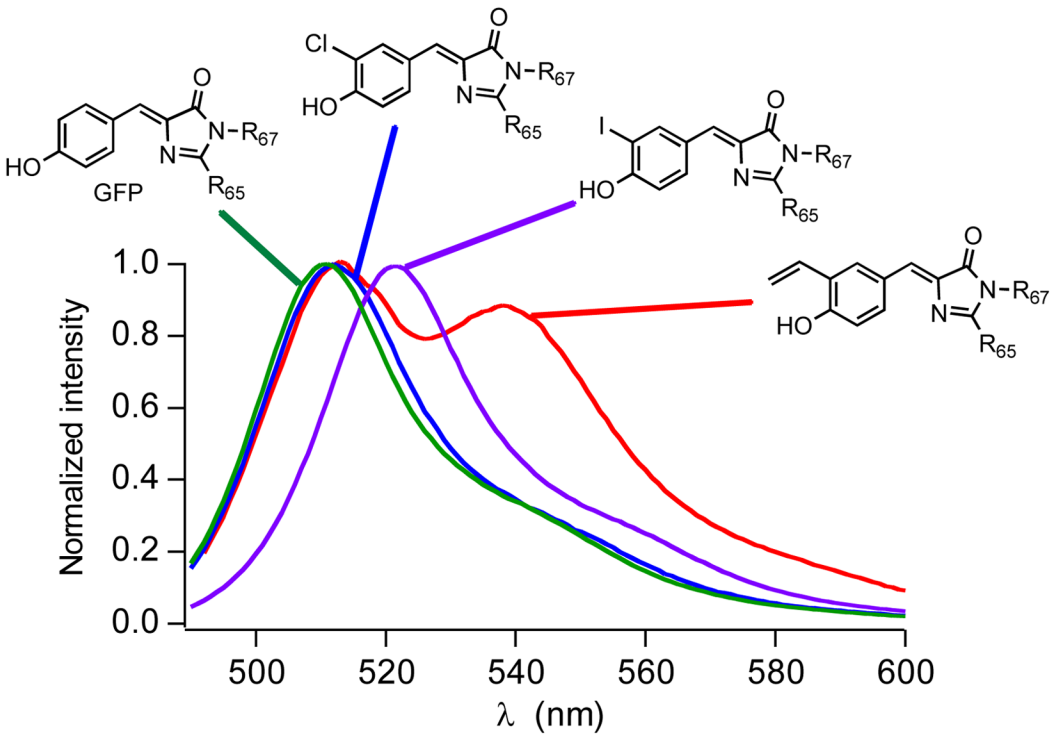


Figure 1.

Normalized fluorescence spectra of GFP and modified GFP containing unnatural amino acids **2**, **3**, and **6** in phosphate buffer solution solution (0.1 M NaCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) after excitation at 480 nm.

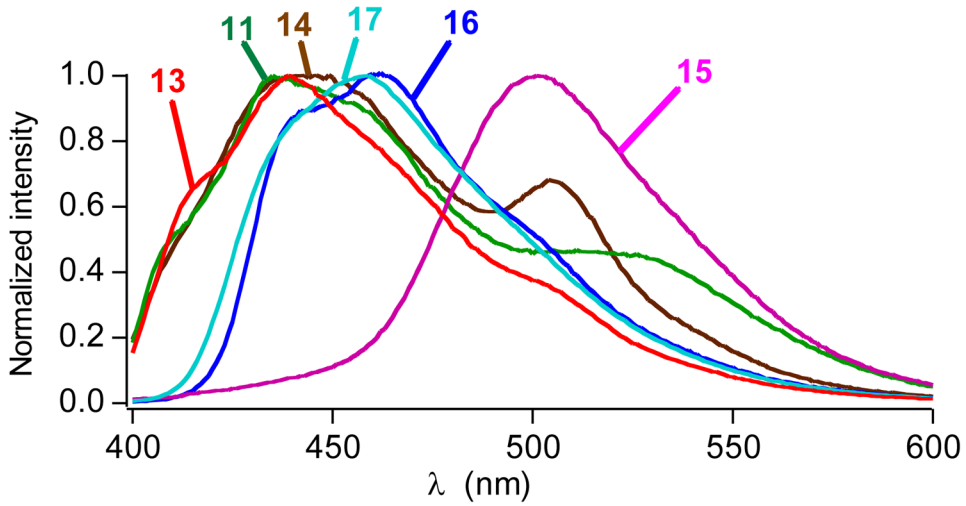
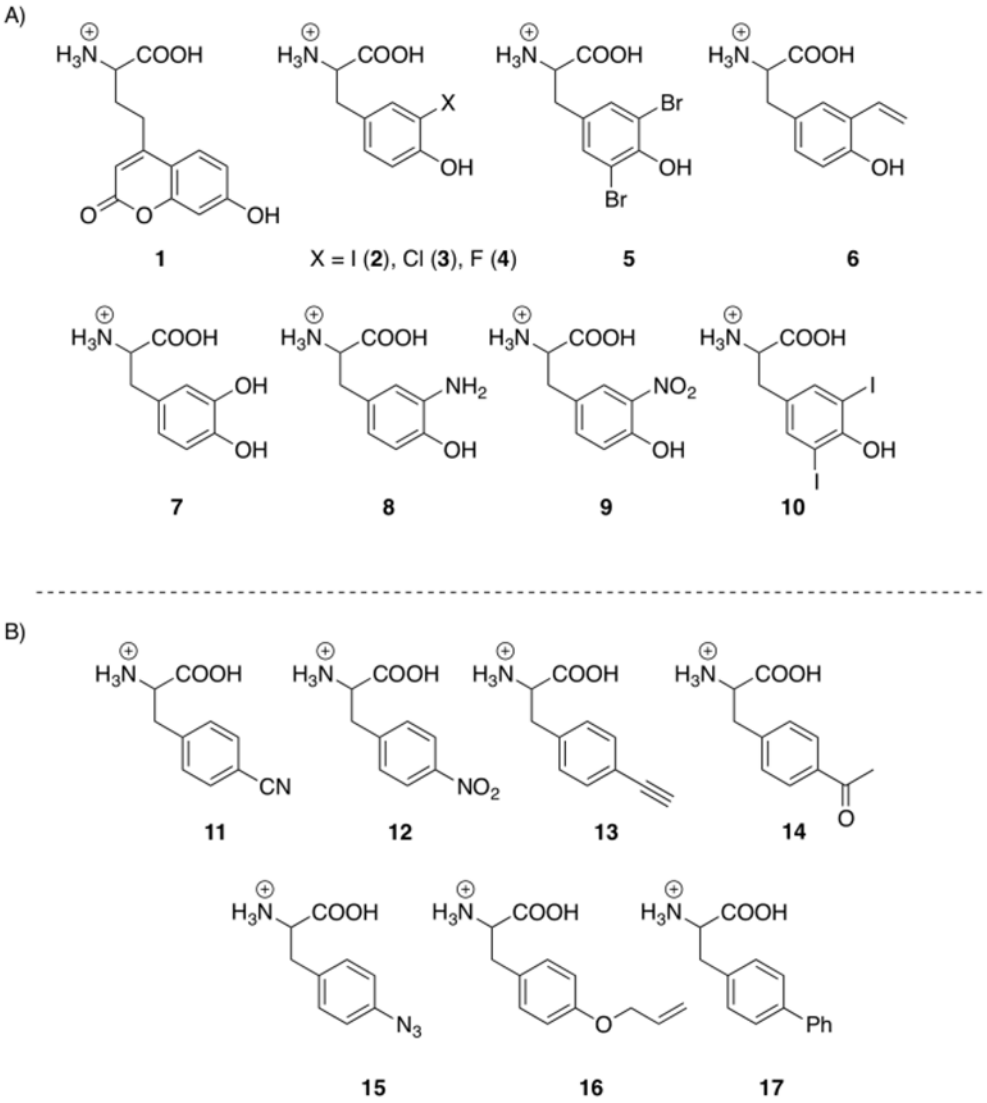


Figure 2.
Normalized fluorescence spectra of modified GFP containing unnatural amino acids in phosphate buffer solution (0.1 M NaCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) after excitation at 365 nm.



Scheme 1.

Table 1

Unnatural amino acid incorporation by the CouARS

Synthetase	UAA	Expected[a]	Observed[b]	Relative % Incorporation[c]
WT	Phe	18352	18353	100 ± 8.2
CouA	**2**	18495	18496	27 ± 2.6
CouA	**3**	18404	18405	19 ± 1.9
CouA	**4**	18387	18387	9.7 ± 0.8
CouA	**5**	18528	18527	10.1 ± 1.4
CouA	**6**	18395	18396	17.5 ± 2.1

[a] LC/MS expected mass for UAA incorporated myoglobin.

[b] Determined by ESI and deconvolution with ChemStation Software (Rev. B.03.02)

[c] Based on GFP-Y151TAG expression, and normalized to WT GFP expression.

Table 2

Fluorescent properties of GFP_{TAG66} Mutants

Synthetase	UAA	λ_{max} (nm)	ϕ_f
*p*CNF	**11**	442	0.039
*p*CNF	**12**	--	≤ 0.005
*p*CNF	**13**	439	0.038
*p*CNF	**14**	435	0.035
*p*CNF	**15**	501	0.11
*p*CNF	**16**	461	0.036
*p*CNF	**17**	459	0.066
CouA	**2**	520	0.43
CouA	**3**	512	0.45
CouA	**6**	538	0.53