Biosynthesis of proteins with novel side chain functionalities

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The incorporation of non-canonical amino acids into (bio)-synthetic proteins has been a long-standing effort in protein chemistry. In principle, this approach offers novel biomolecular reagents for biophysical, structural or biochemical research as well as biotechnological and pharmaceutical applications. A versatile method for the site-specific incorporation of non-natural amino acids must exploit a nucleic acid codon that is not actively used by the genetic code. Thus, the amber stop codon (UAG), which is also subject to natural nonsense suppression mechanisms, has been exploited to create an additional coding triplet. To adapt this approach to the overexpression of proteins in live host cells, an orthogonal aminoacyl-tRNA synthetase (aaRS) with novel substrate specificity, together with an alien suppressor tRNA, is instrumental.

We have developed a new strategy for the rapid selection of mutant aaRSs with specificities for non-natural amino acids based on fluorescence-activated cell sorting (FACS) of *E. coli* using the enhanced green fluorescent protein (eGFP) equipped with an internal amber stop codon as reporter. A one-plasmid expression system was established encoding the modified *M. jannaschii* TyrRS, a cognate suppressor tRNA, and $eGFP_{UAG}$ in an individually regulatable fashion. Using this system, a previously described aaRS with specificity for MeTyr was engineered for ten-fold improved incorporation of the foreign amino acid by focused mutagenesis and applying alternating cycles of positive and negative FACS selection in the presence or absence, respectively, of MeTyr. This system was also used to engineer an aaRS/tRNA pair with unprecedented activity towards *p*-acetyl-phenylalanine (Apa) and it was adapted to the pyrrolysyl-tRNA-Synthetase (PylRS) from *M. barkeri*, an alternative orthogonal aaRS/tRNA pair that shows advantages with regard to foreign amino acid specificity and amber suppression efficiency.

The optimized synthetases were employed for the preparation of a modified uvGFP carrying MeTyr as part of its fluorophore and for the site-specific incorporation of the fluorescent non-natural amino acid L-(7-hydroxycoumarin-4-yl)ethylglycine into the enhanced cyan fluorescent protein (eCFP) at a permissive surface position ~20 Å away from the endogenous fluorophore. The resulting $eCFP^{Cou}$ reveals almost quantitative intramolecular Förster transfer (FRET) between its two fluorophores and shows unprecedented spectroscopic properties with an extreme apparent Stokes shift of about 110 nm. Apa was efficiently introduced into an Anticalin directed against a biomedical marker of tumour angiogenesis, offering the possibility for site-specific labelling with fluorophores, toxins or radiometal chelators due to its unique chemical reactivity with various aminoxy-derivatized compounds. Taken together, our combined genetic/selection system greatly facilitates the engineering and optimization of orthogonal aaRS/tRNA pairs to incorporate novel non-natural amino acids into recombinant proteins at preparative scale.