

4cn Protein Engineering by Single or Multiple Site-Specific Incorporation of Nonnatural Amino Acids *in Vivo*

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Site-specific incorporation of nonnatural amino acids containing novel functional groups into recombinant proteins provides new chemical and physical tools for protein engineering. Single-site insertion of a novel functional group in response to an amber stop codon has been realized by introduction of a twenty-first transfer RNA (tRNA)/ aminoacyl-tRNA synthetase pair into *E. coli*. Reactive functional groups including azide, bromo and iodo groups allow bio-orthogonal protein chemistry that cannot be obtained with natural amino acids. This strategy relies on single-site insertion at a stop codon, and it is limited by modest suppression efficiency. In an alternative approach, we have validated the hypothesis that one of the degenerate phenylalanine codons can be re-assigned to a nonnatural amino acid in appropriately engineered strains of *E. coli*. The concept of breaking the degeneracy of the genetic code has been expanded to degenerate leucine codons, although codon re-assignment is not yet complete. The method presented here will find diverse applications including glycosylation, pegylation and immobilization of proteins.