Chapter 1

INTRODUCTION

Portions of this text are taken from:

Connor, R.E. and D.A. Tirrell, *Non-canonical amino acids in protein polymer design.* Polymer Reviews, 2007. **47**(1): p. 9-28

1.1 Protein Biosynthesis

An elegant hereditary informational system is evolutionarily conserved throughout all organisms to precisely synthesize proteins in a timely and controlled manner. Proteins are composed of linear chains of amino acids which fold into three-dimensional structures to form active species. The basic genetic material, DNA, encodes the amino acid sequence of the protein; that sequence is transcribed into a messenger RNA (mRNA) molecule from the DNA, and finally, the mRNA is decoded by transfer RNA (tRNA) during processing by the ribosome. The tRNAs bind to a particular sequence of three nucleotides within the mRNA (codons), and their correct transfer of amino acids maintains the desired sequence in the nascent polypeptide. This central dogma of biology is illustrated in Figure 1-1.

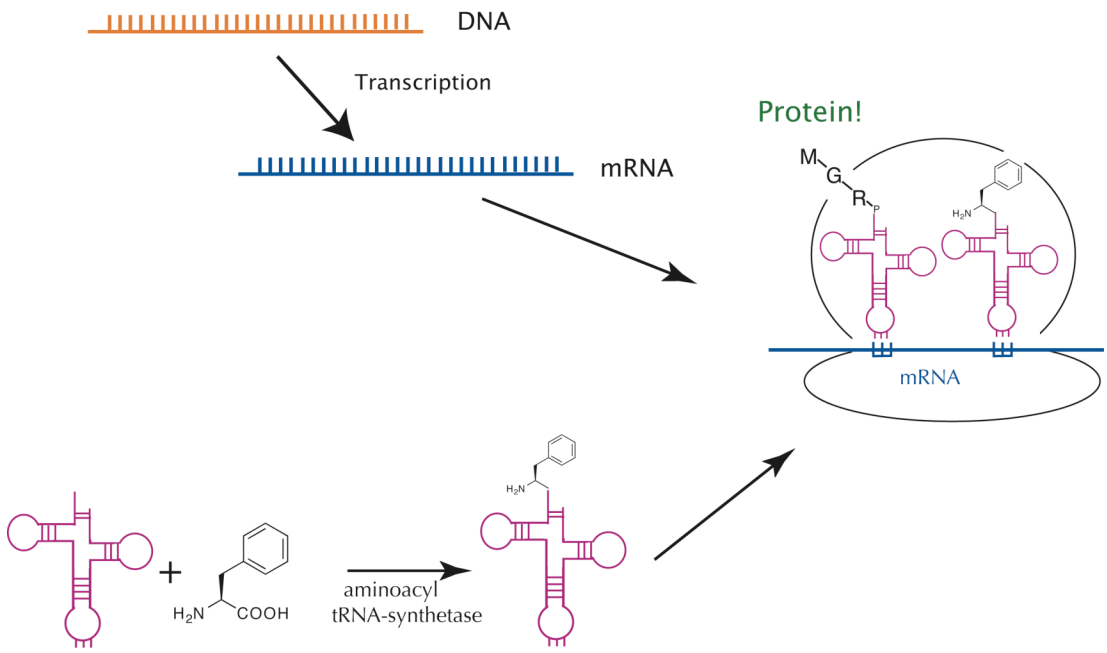

Figure 1-1. Diagram depicting the central dogma of biology. DNA is transcribed to mRNA. mRNA is shuttled to the ribosome and decoded by tRNAs bearing an amino acid for extension of the protein chain.

High fidelity of protein synthesis is due not only to high affinity recognition of the a codon by the correct tRNA, but also the correct addition of amino acid to cognate tRNA. The fidelity of the latter function is ensured by the enzymes that form the aminoacyl-tRNA conjugate, the aminoacyl-tRNA synthetases. In most organisms, every amino acid is appended to its cognate tRNA by a single synthetase, and these enzymes discriminate among the twenty naturally occurring amino acids through active site recognition. Some aminoacyl-tRNA synthetases have another level of quality control; post-transfer editing domains remove amino acids that have been incorrectly acylated to the substrate tRNA.[1, 2]

Despite the safeguards designed for high fidelity of protein synthesis in all orgnisms, evidence for the infiltration of the protein synthesis machinery of bacteria by amino acid analogs was first noted in the 1950s.[3, 4] During the 1960s, the incorporation of 5,5,5-trifluoroleucine, p-fluorophenylalanine, azatryptazan, canavanine, 4-methyltryptophan, 2-thienylalanne, and m-fluorotyrosine into proteins in *E. coli* was accomplished. In the last 30 years, biochemical interest in harnassing the new properties of non-canonical amino acids for protein engineering has increased and led to a variety of methods to introduce them into proteins.

1.2 Methods for the introduction of non-natural amino acids to proteins

1.2.1 Chemical Aminoacylation of tRNA

In the late 1970s, Sidney Hecht developed a groundbreaking method for chemically aminoacylating tRNA molecules.[5] The use of RNA ligase to conjugate an acylated dinucleotide to an abbreviated tRNA enabled the introduction of non-natural

amino acids into proteins using *in vitro* translational systems. The chemical acylation of natural tRNAs allowed global incorporation of a photoreactive amino acid analog in place of a natural one throughout a protein synthesized *in vitro*.[6] One limitation of this method is that a tRNA-free cellular extract is required; otherwise, the chemically synthesized tRNAs are forced to compete with naturally charged tRNAs for ribosomal occupation. This method of replacement *in vitro* has recently been extended by Sando *et al* to remove the requirement for tRNA-free translational systems. They reduced the *in vitro* aminoacylation of a target tRNA through small molecule inhibition of the tRNA synthetase, thus allowing complete replacement of a natural amino acid without using a tRNA-free translation system.[7]

In order to circumvent competition for ribosomal insertion and to allow the introduction of a 21st amino acid with the twenty natural amino acids represented in protein synthesis, researchers turned to suppressor tRNAs.[8, 9] Within the genetic code, three stop codons, known as the *amber*, *opal* and *ochre* codons, signal the termination of protein synthesis. In some strains of bacteria, tRNAs have been identified that recognize *amber* codons and insert an amino acid, often glutamine or selenocysteine, rather than halting translation. These tRNAs are considered "suppressors" because they prevent or suppress the termination of protein synthesis in the ribosome. Recognizing this feature, a number of research groups have sought to reassign these stop codons to non-natural amino acids by mis-acylation of suppressor tRNAs. These tRNAs have no natural cognate tRNA to compete for codon recognition during protein synthesis.[9] The use of chemically aminoacylated suppressor tRNA to infiltrate translation in cell-free systems,

although limited in the production of protein, has been effectively used to study detailed aspects of protein biochemistry through a variety of analytical methods.[8, 10]

The reassignment of amber stop codons in proteins through chemically mis-acylated tRNA molecules has also been extended from *in vitro* to *in vivo* methods through the use of microinjection into living cells. In the Dougherty lab, suppression with microinjected *Tetrahymena* tRNA in *Xenopus* oocytes was used to probe the biophysical and structural properties of expressed ion channels.[11]

An alternative to the recognition of a stop codon by a mis-acylated tRNA for site-specific incorporation is frameshift interpretation by a mis-acylated suppressor tRNA. Sisido, Hohsaka and others have developed a number of suppressor tRNAs that will recognize four- and five-base codons, instead of the normal three-base ones.[12, 13] These methods require the removal of any natural occurrences of the four base sequence to prevent unwanted insertion of the non-natural amino acid into the protein sequence. A variety of interesting amino acids have been introduced with these methods, particularly fluorescent amino acids such as 2,6-dansyl-aminophenylalanine and 2-acridonylamine.[14, 15]

1.2.2 Enzymatic aminoacylation and site-specific incorporation into proteins

One of the limitations of chemical aminoacylation is the effort required to produce a large quantity of aminoacylated-tRNA. As such, *in vivo* enzymatic aminoacylation of non-natural amino acids and subsequent incorporation through the native translational machinery provides an alternative route for increased production of modified proteins. However, the aminoacylation of non-natural amino acids is limited by

the permissivity of tRNA synthetases and, at the same time, is forced to compete with the cognate amino acid itself for synthetase activity. To circumvent substrate competition, an orthogonal tRNA and synthetase pair that will not interact with the native synthetases and tRNAs can be introduced. This orthogonal tRNA can then be used to reassign a suppressor codon for the non-natural amino acid. This type of reassignment was first accomplished by Furter using an orthogonal phenylalanyl-tRNA synthetase (PheRS) and $tRNA^{phe}$ pair from *S. cerevisiae* in *E. coli* to incorporate *p*-fluorophenylalanine site-specifically into proteins. The non-canonical amino acid was incorporated into murine dihydrofolate reductase (mDHFR), a highly expressed model protein, at an amber stop codon.[16] Background misincorporation from the endogenous *E. coli* PheRS was reduced by its inability to acylate *p*-fluorophenylalanine due to a mutation at residue 294 that reduces the size of the active site.[17] Phenylalanine was replaced with the fluorinated analog at the amber codon in 64-75% of the protein produced with this system.

In order to improve the fidelity of the *in vivo* approach, Schultz and coworkers have developed powerful screening methods to engineer mutant synthetases for site-specific introduction of a wide variety of non-natural amino acids via nonsense suppression.[18] Positive selection relies on the ability of the desired mutant synthetase to incorporate the target non-natural amino acid and allow suppression of an amber codon in an antibiotic resistance gene. The sustained translation of chloramphenicol acetyltransferase (CAT) in the presence of the antibiotic chloramphenicol allows the host cell of the mutant synthetase to survive. The mutant synthetases surviving the positive screen were then transferred into a strain containing barnase, a lethal gene that encodes a

ribonuclease. This copy of barnase contains an amber stop codon that if suppressed, allows the production of active enzyme. Thus, a synthetase that charges a natural amino acid onto the suppressor tRNA will kill its host cell. Multiple rounds of screening will generate a synthetase mutant selective for a non-natural amino acid in the presence of natural amino acids. Introduction of *o*-methyltyrosine into DHFR was achieved for this method and has been adapted to identify synthetases for incorporation of more than thirty amino acids *in vivo*.[18, 19] Subsequent screens derived from this method have also relied upon different techniques, including phage display and high-throughput flow cytometry screens for selective synthetases.[19]

1.2.3 Replacement of natural amino acids with non-natural surrogates

Despite the advances in site-specific placement of non-natural amino acids *in vivo*, the production of large quantities of protein remains a challenge. The global introduction of a novel chemical moiety will not only affect bulk material characteristics, but also achieve production yields close to wild-type levels of protein expression in microbial hosts.[20] This method follows from the classic amino acid replacement experiments from the 1950s.[3, 4, 21-23] Essentially, bacteria in a culture grown in minimal medium are deprived of an amino acid upon induction of expression of a target protein. The non-natural amino acid is then added to the medium and it is utilized in place of the missing natural substrate by the tRNA synthetase. This method specifically exploits the permissivity of both the tRNA synthetase and the ribosomal apparatus of the microbial host. With this method, the extent of incorporation can be enhanced by washing the cells before induction to remove any residual natural amino acid.[11, 17, 24,

25] The global replacement of a natural amino acid can also be achieved through fermentation, which allows the growth of cultures to higher cell densities and therefore higher protein yields. The fermentation method, also called the selective pressure incorporation (SPI) method, requires that the bacteria survive in the presence of the non-natural amino acid. The cultures are grown to mid-log phase in media containing limited amounts of the cognate amino acid, and once that amount is thought to be depleted, the addition of the non-canonical amino acid and induction of expression with IPTG are instigated simultaneously.[20] Both of these methods can be readily extended to a more diverse set of analogous molecules by concomitant over-expression of either a natural or modified synthetase.[26-30] In 1990, Hendrickson and Ogata revisited the classic selenium replacement experiments by Cowie and Cohen to solve the phase problem in x-ray crystallography using multiwavelength anomalous diffraction.[3, 25] This method has contributed heavily to the abundance of crystal structure elucidation in the last 15 years.[31]

1.3 Protein degradation and the N-end rule pathway

The control of biological processes by proteins is not limited to their catalysis of necessary reactions, but also by their temporal and spatial activity. A subtle method for the control of protein activity is through constant degradation of the active enzyme, the cessation of which allows a rapid burst of protein activity at a designated time and place.[32] Control of protein activity through degradation has been observed in prokaryotes for signaling of morphological changes during cell cycle progression and for responses to environmental stresses such as heat shock.[33, 34] One pathway for

processive or complete protein degradation is the N-end rule pathway, which is found in both prokaryotes and eukaryotes. The N-end rule states that the half-life of any given protein is dictated by its N-terminal residue.[35, 36] Essentially, the N-terminal residue of a protein can be recognized as a signal for degradation by chaperone proteins (recognins) who will shuttle it to an ATP-dependent protease for processive degradation. The N-terminal residue that is directly recognized and induces degradation without further modification is considered a primary destabilizing residue. In mammals and yeast, the primary destabilizing residues are lysine and arginine (Figure 1-2), while in prokaryotes, the primary destabilizing residues are leucine, phenylalanine, tyrosine, and tryptophan.[37, 38] In addition to the primary destabilizing residues, proteins containing a "secondary destabilizing residue" at the N-terminus are modified with a primary destabilizing residue to induce degradation. This N-terminal modification is performed by aminoacyl-tRNA protein transferases, which can convey amino acids from an aminoacyl-tRNA to a protein.[39, 40] These transferases are found in both prokaryotes and eukaryotes, although with slightly variant specificities, as expected from the difference in primary destabilizing residues (Figure 1-2). In mammals and yeast, the arginyl-transferase appends arginine to the N-terminus of proteins bearing oxidized cysteine, glutamic or aspartic acid, the secondary destabilizing residues.

In prokaryotes, leucine and phenylalanine are appended to the N-termini of proteins bearing secondary destabilizing residues by enzymes called leucyl, phenylalanyl-transferases. Some bacteria, such as *Vibrio vulnificus*, harbor two separate transferases that recognize different N-terminal residues on their substrates, but both append the primary destabilizing residue, leucine (Figure 1-2).[41] In *E. coli*, only the L,F-

transferase encoded by the *aat* gene (denoted as Aat in this chapter) is present. The specificity of Aat towards its aminoacyl substrates is permissive and it can transfer not only leucine, but also phenylalanine and methionine from aminoacyl-tRNA to a protein substrate *in vivo*.[42-44] In *E. coli*, the N-end rule recognin is hypothesized to be solely the ClpS chaperone protein; however, some evidence has shown that ClpS is not required for the degradation of N-end rule substrates.[45, 46] The ClpAP protease complex is required for processing N-end rule substrates, as demonstrated by the disruption of the degradation pathway in *clpA* knockout strains.[47] Despite the elegant simplicity of the N-end rule pathway in *E. coli*, no substrates of Aat or unmodified degrons of the pathway have been identified to date. As Bernd Bukau writes, "The N-end rule pathway has not lost its glamour and will still surprise with new twists and turns[48]."

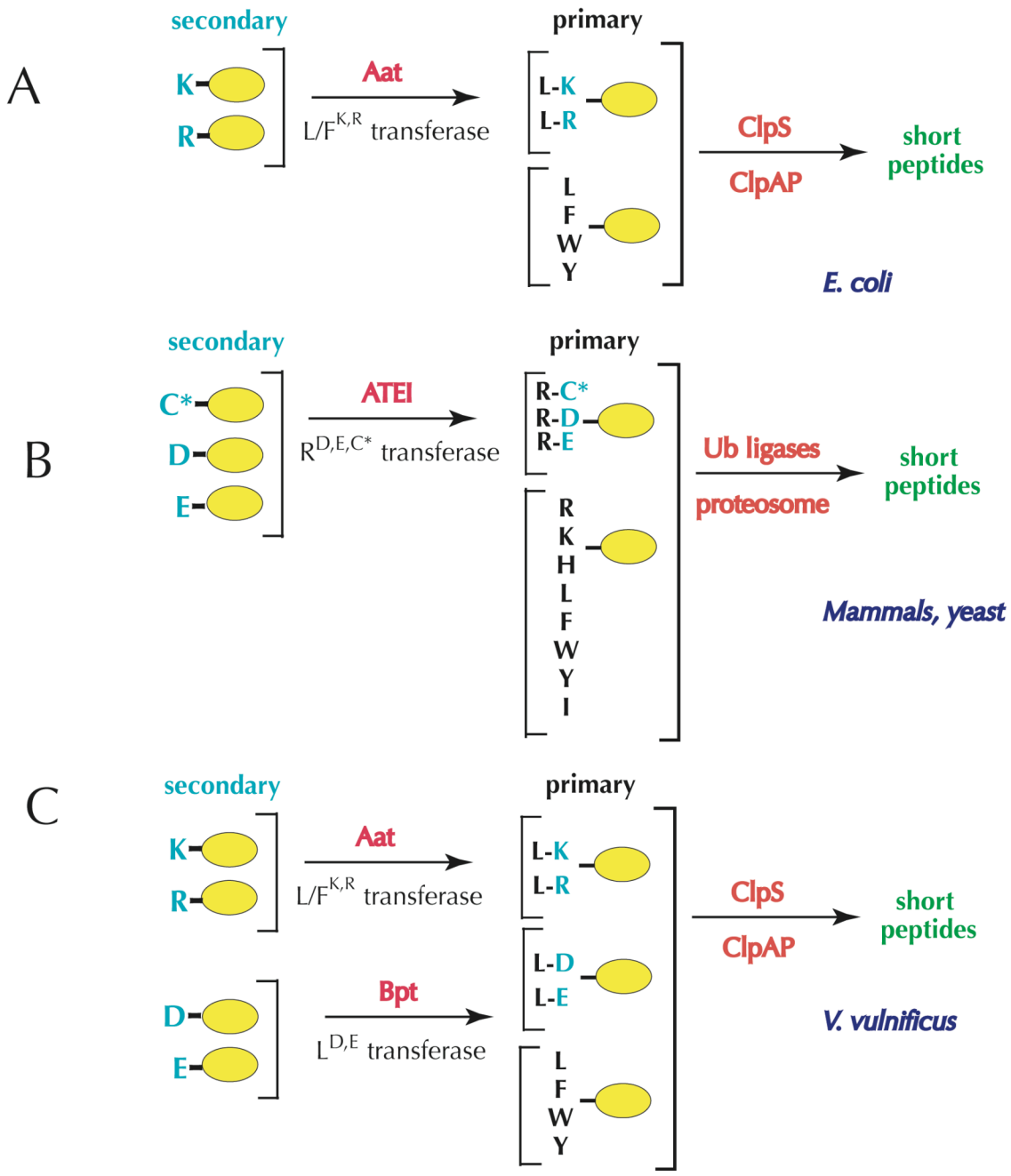

Figure 1-2. Diagram of the N-end rule pathway. Adapted from Graciet *et al., PNAS,* 2006, **103**(9): p3078-3083. The N-end rule pathway in A) *E. coli*, B) mammals and yeast, and C) *V. vulnificus.*

1.4 Outline of thesis

In this thesis, a new method for the *in vitro* post-translational modification of proteins with non-canonical amino acids using the *E. coli* enzyme, the L,F-transferase, is developed. An analytical method for the evaluation of amino acids as candidate aminoacyl-tRNA substrates of the L,F-transferase is described using a peptide-aminocoumarin conjugate as the acceptor protein. The product is a tripeptide aminocoumarin that can be separated from the substrate by HPLC and verified by electrospray ionization mass spectrometry. Twenty-seven different non-canonical amino acids are evaluated as aminoacyl substrates of the L,F-transferase; twenty-four of them are transferred. The results of the peptide modification assay can be extended to the *in vitro* modification of a model protein. Five reactive analogs are appended to *E. coli* dihydrofolate reductase bearing an arginine N-terminus *in vitro.* The utility of the method for the creation of protein bioconjugates was demonstrated by the addition of *p*-ethynylphenylalanine to the N-terminus of the model protein for ligation to both a fluorescein-polyethylene-glycol conjugate and a biotin affinity tag. The total yield of modified protein was found to be above 80% after addition of the amino acid and ligation to the polymer using copper-catalyzed [3+2] azide-alkyne cycloaddition.

Progress towards the extension of the *in vitro* method to an *in vivo* modification system was made. A screen was designed to identify a mutant leucyl, phenylalanyl-tRNA transferase selective for the desired non-canonical amino acid, *p*-ethynylphenylalanine. The L,F transferase gene in the host *E. coli* strains was removed by chromosomal recombination, and a library of mutant L,F-transferases was

synthesized. A DNA construct encoding a protein fusion of ubiquitin and green fluorescent protein that will be used as a reporter in the screen was also produced.

M13 bacteriophage were produced with coat proteins bearing a methionine analogue, azidohomoalanine, which was introduced through codon reassignment during coat protein over-expression. The resultant Aha-bearing phage remained infectious and were modified with alkyne tags using either copper-catalyzed or strain-catalyzed [3+2] cycloaddition. After copper-catalyzed modification, the phage lost infectivity; however, strain-catalyzed modification was not deleterious to phage infection.

1.5 References

1. Tang, Y. and D.A. Tirrell, *Attenuation of the editing activity of the Escherichia coli Leucyl-tRNA synthetase allows incorporation of novel amino acids into proteins in vivo.* Biochemistry, 2002. **41**(34): p. 10635-10645.

2. Mursinna, R.S., T.L. Lincecum, and S.A. Martinis, *A conserved threonine within Escherichia coli leucyl-tRNA synthetase prevents hydrolytic editing of leucyl-tRNA(Leu).* Biochemistry, 2001. **40**(18): p. 5376-5381.

3. Cowie, D.B. and G.N. Cohen, *Biosynthesis by Escherichia-Coli of Active Altered Proteins Containing Selenium Instead of Sulfur.* Biochimica Et Biophysica Acta, 1957. **26**(2): p. 252-261.

4. Brawerman, G. and M. Ycas, *Incorporation of the Amino Acid Analog Tryptazan into the Protein of Escherichia-Coli.* Archives of Biochemistry and Biophysics, 1957. **68**(1): p. 112-117.

5. Hecht, S.M., et al., *Chemical Aminoacylation of Transfer-RNAs.* Journal of Biological Chemistry, 1978. **253**(13): p. 4517-4520.

6. Baldini, G., et al., *Mischarging Escherichia-Coli Transfer Rnaphe with L-4'-[3-(Trifluoromethyl)-3h-Diazirin-3-Yl]Phenylalanine, a Photoactivatable Analog of Phenylalanine.* Biochemistry, 1988. **27**(20): p. 7951-7959.

7. Sando, S., et al., *A small-molecule-based approach to sense codon-templated natural-unnatural hybrid peptides. Selective silencing and reassignment of the sense codon by orthogonal reacylation stalling at the single-codon level.* Journal of the American Chemical Society, 2005. **127**(22): p. 7998-7999.

8. Ellman, J., et al., *Biosynthetic Method for Introducing Unnatural Amino-Acids Site-Specifically into Proteins.* Methods in Enzymology, 1991. **202**: p. 301-336.

9. Bain, J.D., et al., *Biosynthetic Site-Specific Incorporation of a Non-Natural Amino-Acid into a Polypeptide.* Journal of the American Chemical Society, 1989. **111**(20): p. 8013-8014.

10. Cornish, V.W. and P.G. Schultz, *A New Tool for Studying Protein-Structure and Function.* Current Opinion in Structural Biology, 1994. **4**(4): p. 601-607.

11. Dougherty, M.J., et al., *Synthesis of a Genetically Engineered Repetitive Polypeptide Containing Periodic Selenomethionine Residues.* Macromolecules, 1993. **26**(7): p. 1779-1781.

12. Hohsaka, T., et al., *Five-base codons for incorporation of nonnatural amino acids into proteins.* Nucleic Acids Research, 2001. **29**(17): p. 3646-3651.

13. Sisido, M., et al., *Four-base codon/anticodon strategy and non-enzymatic aminoacylation for protein engineering with non-natural amino acids.* Methods, 2005. **36**(3): p. 270-8.

14. Hohsaka, T., et al., *Position-specific incorporation of dansylated non-natural amino acids into streptavidin by using a four-base codon.* Febs Letters, 2004. **560**(1-3): p. 173-177.

15. Hamada, H., et al., *Position-specific incorporation of a highly photodurable and blue-laser excitable fluorescent amino acid into proteins for fluorescence sensing.* Bioorganic & Medicinal Chemistry, 2005. **13**(10): p. 3379-3384.

16. Furter, R., *Expansion of the genetic code: Site-directed p-fluoro-phenylalanine incorporation in Escherichia coli.* Protein Science, 1998. **7**(2): p. 419-426.

17. Kast, P. and H. Hennecke, *Amino-Acid Substrate-Specificity of Escherichia-Coli Phenylalanyl-Transfer Rna-Synthetase Altered by Distinct Mutations.* Journal of Molecular Biology, 1991. **222**(1): p. 99-124.

18. Wang, L., et al., *Expanding the genetic code of Escherichia coli.* Science, 2001. **292**(5516): p. 498-500.

19. Wang, L. and P.G. Schultz, *Expanding the genetic code.* Angewandte Chemie-International Edition, 2005. **44**(1): p. 34-66.

20. Budisa, N., *Prolegomena to future experimental efforts on genetic code engineering by expanding its amino acid repertoire.* Angewandte Chemie-International Edition, 2004. **43**(47): p. 6426-63.

21. Rennert, O.M. and H.S. Anker, *On Incorporation of 5',5',5'-Trifluoroleucine into Proteins of E. coli.* Biochemistry, 1963. **2**(3): p. 471-476.

22. Richmond, M.H., *Effect of Amino Acid Analogues on Growth and Protein Synthesis in Microorganisms.* Bacteriological Reviews, 1962. **26**(4): p. 398.

23. Munier, R. and G.N. Cohen, *Incorporation of Structural Analogues of Amino Acid into the Bacterial Proteins During Their Synthesis In Vivo.* Biochimica Et Biophysica Acta, 1959. **31**(2): p. 378-391.

24. Ibba, M. and H. Hennecke, *Relaxing the Substrate-Specificity of an Aminoacyl-Transfer-Rna Synthetase Allows in-Vitro and in-Vivo Synthesis of Proteins Containing Unnatural Amino-Acids.* Febs Letters, 1995. **364**(3): p. 272-275.

25. Hendrickson, W.A., J.R. Horton, and D.M. Lemaster, *Selenomethionyl Proteins Produced for Analysis by Multiwavelength Anomalous Diffraction (MAD) - a Vehicle for Direct Determination of 3-Dimensional Structure.* Embo Journal, 1990. **9**(5): p. 1665-1672.

26. Tang, Y. and D.A. Tirrell, *Biosynthesis of a highly stable coiled-coil protein containing hexafluoroleucine in an engineered bacterial host.* Journal of the American Chemical Society, 2001. **123**(44): p. 11089-11090.

27. Kirshenbaum, K., I.S. Carrico, and D.A. Tirrell, *Biosynthesis of proteins incorporating a versatile set of phenylalanine analogues.* Chembiochem, 2002. **3**(2-3): p. 235-237.

28. Kiick, K.L., J.C.M. van Hest, and D.A. Tirrell, *Expanding the scope of protein biosynthesis by altering the methionyl-tRNA synthetase activity of a bacterial expression host.* Angewandte Chemie-International Edition, 2000. **39**(12): p. 2148-+.

29. Kiick, K.L. and D.A. Tirrell, *Protein engineering by in vivo incorporation of non-natural amino acids: Control of incorporation of methionine analogues by methionyl-tRNA synthetase.* Tetrahedron, 2000. **56**(48): p. 9487-9493.

30. Datta, D., et al., *A designed phenylalanyl-tRNA synthetase variant allows efficient in vivo incorporation of aryl ketone functionality into proteins.* Journal of the American Chemical Society, 2002. **124**(20): p. 5652-5653.

31. Hendrickson, W.A. and C.M. Ogata, *Phase determination from multiwavelength anomalous diffraction measurements.* Macromolecular Crystallography A, 1997. **276**: p. 494-523.

32. Jenal, U. and R. Hengge-Aronis, *Regulation by proteolysis in bacterial cells.* Current Opinion in Microbiology, 2003. **6**(2): p. 163-72.

33. Domian, I.J., A. Reisenauer, and L. Shapiro, *Feedback control of a master bacterial cell-cycle regulator.* Proceedings of the National Academy of Sciences (U. S. A.), 1999. **96**(12): p. 6648-53.

34. Domian, I.J., K.C. Quon, and L. Shapiro, *Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle.* Cell, 1997. **90**(3): p. 415-24.

35. Gonda, D.K., et al., *Universality and structure of the N-end rule.* Journal of Biological Chemistry, 1989. **264**(28): p. 16700-12.

36. Bachmair, A. and A. Varshavsky, *The degradation signal in a short-lived protein.* Cell, 1989. **56**(6): p. 1019-32.

37. Bachmair, A., D. Finley, and A. Varshavsky, *In vivo half-life of a protein is a function of its amino-terminal residue.* Science, 1986. **234**(4773): p. 179-86.

38. Tobias, J.W., et al., *The N-end rule in bacteria.* Science, 1991. **254**: p. 1374-1377.

39. Soffer, R.L., *Aminoacyl-Transfer-Rna Transferases.* Advances in Enzymology and Related Areas of Molecular Biology, 1974. **40**: p. 91-139.

40. Soffer, R.L., *Aminoacyl Transfer Rna-Protein Transferases - Novel Class of Enzymes Catalyzing Peptide Bond Formation.* Transactions of the New York Academy of Sciences, 1970. **32**(8): p. 974-&.

41. Graciet, E., et al., *Aminoacyl-transferases and the N-end rule pathway of prokaryotic/eukaryotic specificity in a human pathogen.* Proceedings of the National Academy of Sciences (U.S.A.), 2006. **103**(9): p. 3078-3083.

42. Scarpulla, R.C., C.E. Deutch, and R.L. Soffer, *Transfer of Methionyl Residues by Leucyl, Phenylalanyl-Transfer-Rna-Protein Transferase.* Biochemical and Biophysical Research Communications, 1976. **71**(2): p. 584-589.

43. Leibowitz, M.J. and R.L. Soffer, *Enzymatic Modification of Proteins .7. Substrate Specificity of Leucyl,Phenylalanyl-Transfer Ribonucleic Acid-Protein Transferase.* Journal of Biological Chemistry, 1971. **246**(17): p. 5207.

44. Leibowitz, M.J. and R.L. Soffer, *Enzymatic Modification of Proteins - Leucyl, Phenylalanyl-Trna-Protein Transferase in Escherichia-Coli.* Federation Proceedings, 1970. **29**(2): p. 899.

45. Erbse, A., et al., *ClpS is an essential component of the N-end rule pathway in Escherichia coli.* Nature, 2006. **439**(7077): p. 753-756.

46. Wang, K.H., R.T. Sauer, and T.A. Baker, *ClpS modulates but is not essential for bacterial N-end rule degradation.* Genes Development, 2007. **21**(4): p. 403-8.

47. Shrader, T.E., J.W. Tobias, and A. Varshavsky, *The N-End Rule in Escherichia-Coli - Cloning and Analysis of the Leucyl, Phenylalanyl-Transfer Rna-Protein Transferase Gene Aat.* Journal of Bacteriology, 1993. **175**(14): p. 4364-4374.

48. Mogk, A., R. Schmidt, and B. Bukau, *The N-end rule pathway for regulated proteolysis: prokaryotic and eukaryotic strategies.* Trends in Cell Biology, 2007. **17**(4): p. 165-72.