Chapter 1

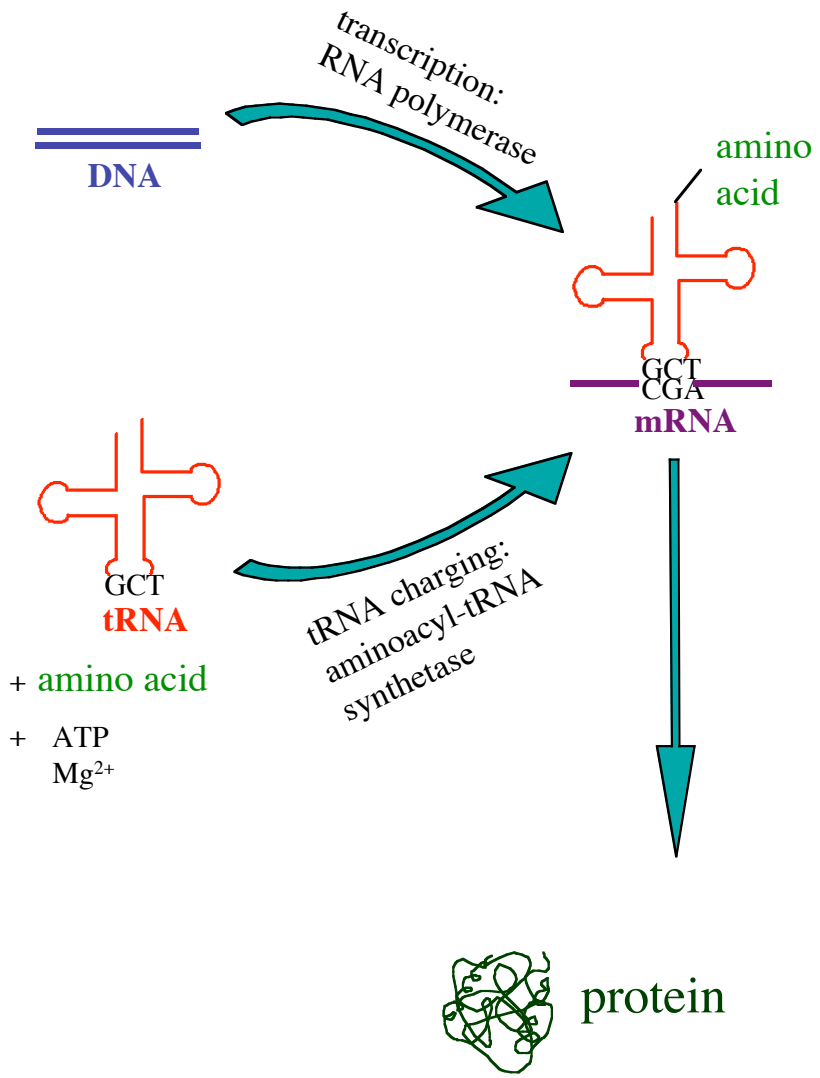
Unnatural Amino Acids in Biomaterials and Protein Engineering

Proteins, which dominate the physiology of all life, are biopolymers comprised of 20 amino acids. Specific structural motifs, generated by complex folding phenomena, allow these macromolecules to accomplish the myriad of tasks needed for life. Post-translational modifications provide further diversification to change not only overall activity, but also spatial and temporal responsiveness [1]. However, in essence proteins are comprised of 20 simple building blocks, many of which are chemically and physically very similar from a chemist's point of view. It is therefore not surprising that a great deal of effort has been directed toward expanding the existing amino acid pool, particularly with moieties distinct from the natural building blocks.

Solid-phase peptide synthesis (SPPS) is a straightforward method for incorporation of unnatural amino acids [2, 3]. SPPS is technically easy and allows the incorporation of any amino acid but is limited by the size of the peptides produced; 50 amino acid peptides can be very challenging to create. However, there are a variety of chemistries, known as chemoselective ligations [4-10], which allow the stitching together of peptide fragments or adding peptides to biosynthetically produced proteins. Recently these techniques, particularly native chemical ligation [11], have allowed the production of large proteins incorporating unnatural amino acids.

A competing focus is based upon subverting the natural biosynthetic machinery to allow incorporation of unnatural amino acids [12-14]. The potential for natural processing of the resultant polypeptides, including folding and post-translational processing, make this approach attractive. Translation, the process of

Figure 1.1. Simplified schematic overview of transcription and translation.



creating polypeptides on messenger RNA templates, is catalyzed by the ribosome. Fidelity in this process is dependent upon the correct pairing of the codon of the messenger RNA and the anticodon of the aminoacylated transfer RNA (figure 1.1). The frequency of error at this step is estimated to be in the order of 10^{-4} [15, 16]. Notably, the codon-anticodon pairing is independent of the nature of the amino acid appended to the tRNA [17]. Naturally many groups have focused on producing misacylated tRNA, which can then be accepted by the ribosome and allow the production any protein containing this amino acid. The Chamberlain and Schultz groups first reported the successful incorporation of unnatural amino acids using cell free translation systems in conjunction with chemically acylated suppressor tRNA (Figure 1.2) [18-21]. Subsequently it was shown that *Xenopus* oocytes, injected with chemically acylated suppressor tRNA and mRNA encoding a target gene with an internal suppression site, could synthesize a target protein bearing the unnatural amino acid site-specifically (Figure 1.3) [22]. The target protein in these studies, nicotinic acetylcholine receptor (nAChR), is ideal because although the technique produces very little protein modern electrophysiology allows the detection of a very small number of active membrane ion channels, attomols of protein are sufficient (Figure 1.4)[23]. This system has allowed the elegant biophysical probing of structure/activity relationships of nAChR [24-27], but highlights the general caveats of chemical acylation for *in vivo* unnatural amino acid incorporation, production and delivery of chemically acylated tRNA and yield of the target protein.

An alternative strategy focuses upon the enzymes responsible for the biosynthesis of aminoacyl-tRNA, a diverse family known as the aminoacyl tRNA

Figure 1.2. Schematic representation of *in vitro* amber suppression.

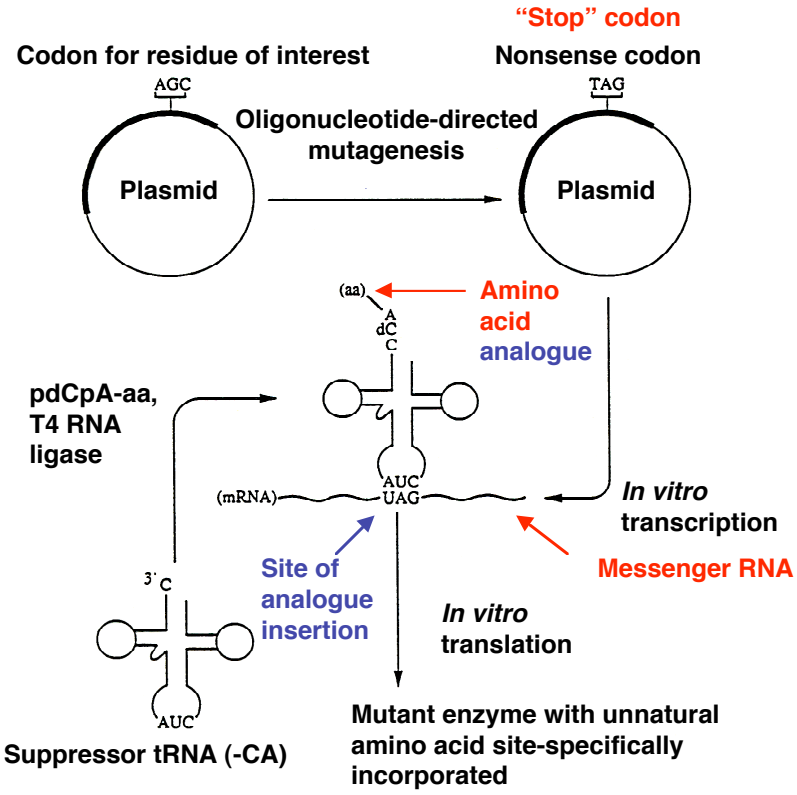


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Figure 1.3. Schematic representation of amber suppression technology used to probe acetylcholine receptor by voltage clamp electrophysiology in *Xenopus* oocytes.

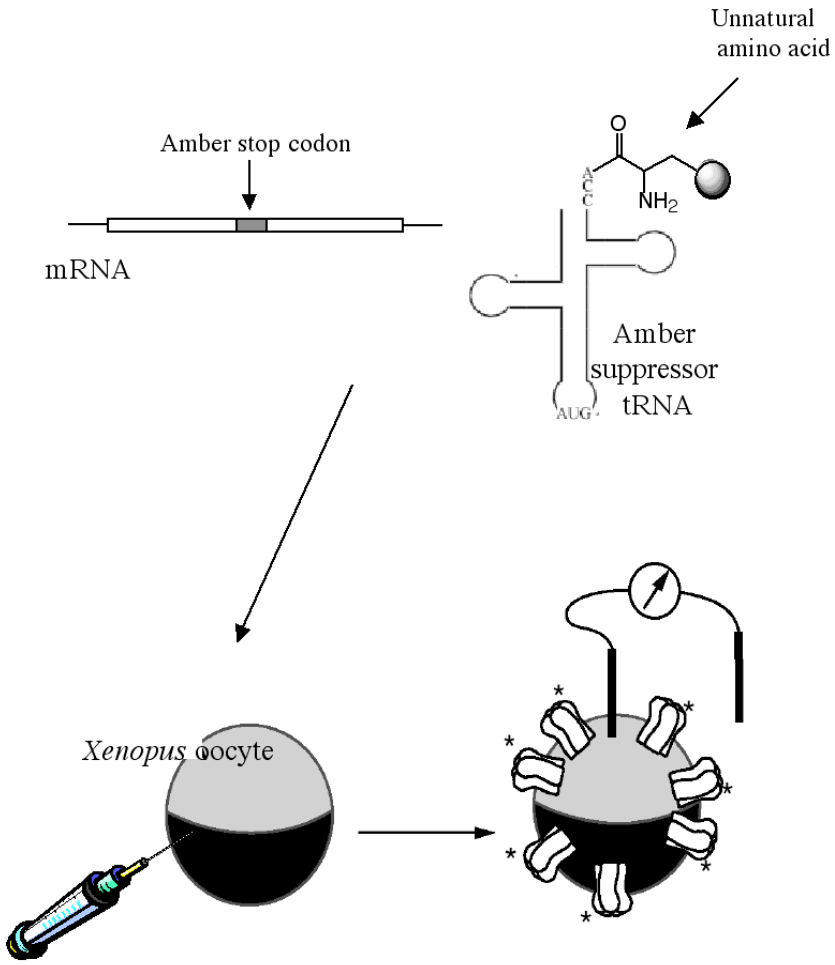
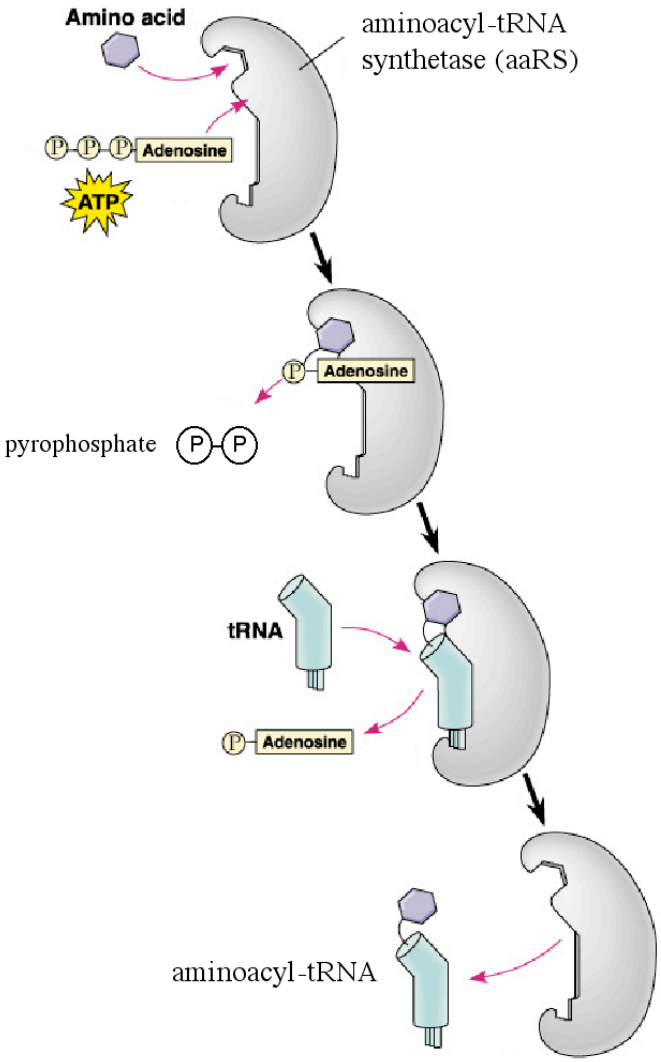


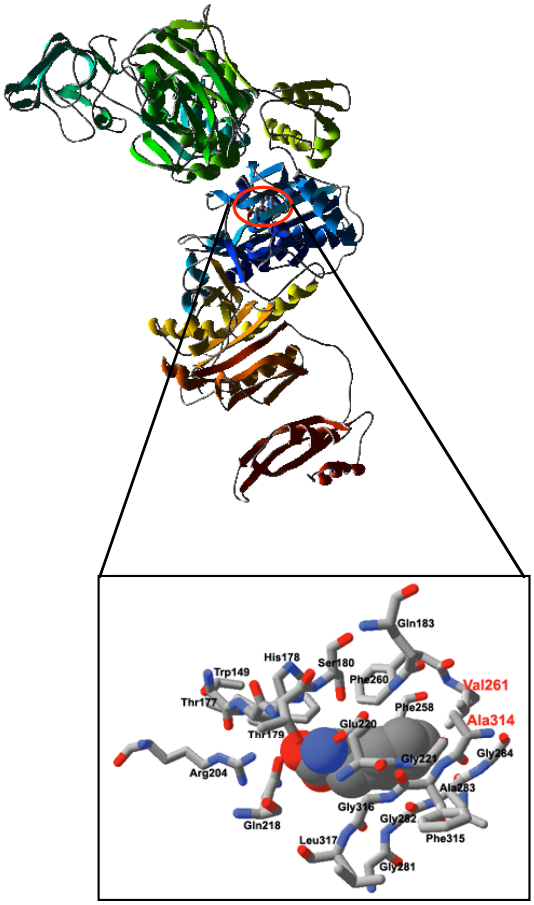
Figure 1.4. Two-step aminoacylation of tRNA catalyzed by aaRS.



synthetases (aaRS). The aaRSs have been intensely studied because of their central role in translation [28-30], as well as their evolutionary importance [31, 32] and involvement in a variety of other processes. Each member of this family catalyzes a two-step reaction. Initially, each amino acid is activated by ATP to form an aminoacyl adenylate. The cognate tRNA(s) then nucleophilically attacks this asymmetric anhydride to form the aminoacyl-tRNA [33] (Figure 1.4). Characteristic motifs and catalytic mechanism divide this family into two parts [34, 35]. The KMSKS and HIGH motifs generally define the class I synthetases, which charge the 2' terminal hydroxyl with the corresponding amino acid. Class II aaRSs exhibit conserved motifs 1,2 and 3 and acylate the 3' hydroxyl, with one exception.

Among Class II aaRSs, phenylalanyl-tRNA synthetase (PheRS) is unique in that it attaches its cognate amino acid, phenylalanine, to the 2'OH of the terminal ribose of the $tRNA^{Phe}$ [36, 37]; further it is an $\alpha_2\beta_2$ hetero-tetrameric enzyme, rather than an α_2 homo-dimer as most of this class of enzymes [38, 39]. The crystal structure of PheRS from *Thermus thermophilus* (PheRS) reveals that the α-subunit is the catalytic unit and the major function for β-subunit is recognition and binding of $tRNA^{Phe}$ (Figure 1.5) [38, 39]. As is characteristic of Class II enzymes, the active site of PheRS is relatively rigid, as revealed by the similar conformations of the ligand-free, Phe-bound and Phe-adenylate analog bound structures of PheRS [39]. Recognition of Phe by PheRS involves hydrogen-bonding interactions with the polar ammonium and carboxylate moieties and multiple van der Waals interactions with hydrophobic side chains. The phenyl ring of substrate Phe is oriented between the hydrophobic side chains of F258 and F260 in the α-subunit, with additional back wall

Figure 1.5. Ribbon representation of the portion of catalytic -subunit of PheRS from *T. thermophilus*. The active site, expanded below, demonstrates bound Phe in space filling model and proximal residues in stick representation.



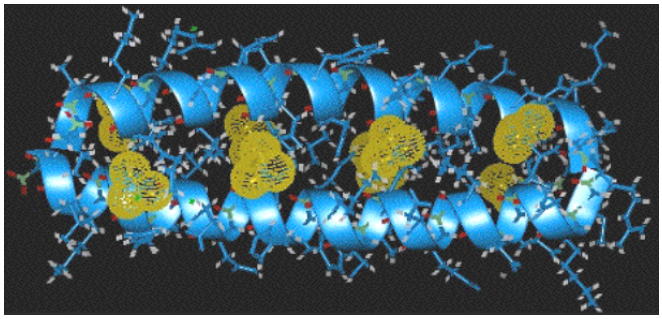
constraints constituted by the side-chains of V261 and A314 in the binding pocket [39].

While the aaRSs have developed mechanisms to prevent the mischarging of natural amino acids, their fidelity wanes in the face of unnatural analogs. This phenomenon has been recognized for decades [40]. Recently, this strategy has been utilized to produce proteins with unnatural physical characteristics. Introduction of fluorinated side chains can stabilize coiled-coil proteins to an extent that would be very difficult to achieve by only canonical amino acids (Figure 1.6) [41, 42]. Ordered protein surfaces displaying trifluoroleucine exhibit a hexadecane contact angle of 70°, in contrast to 17° for the same protein displaying leucine [43] (Figure 1.7). Introduction of amino acid analogs depends upon expression of the target protein in a host auxotrophic for the natural amino acid in the presence of a large amount of the analog of choice. A surprisingly large number of amino acids can be misincorporated applying this technique (Figure 1.8). The range of analogs can be expanded further by overexpression of the aaRS of interest [44-46]. Alternatively, disabling editing functions inherent to some aaRSs can increase the number of accepted analogs [47].

Despite these advances, viable analogs, on the whole, are still limited to those sterically similar to the natural amino acid. Chapter 2 details our efforts to increase the number of analogs that can infiltrate the phenylalanine codon through the use of a known mutant PheRS with an expanded binding pocket. Despite the large number of diverse and chemically interesting analogs this mutant was able to tolerate, it was not able to process *para*-acetylphenylalanine. This analog was particularly interesting because of it would provide access to the ubiquitous ketone coupling chemistry. To

Figure 1.6. Stabilization of coiled coil peptides as a result of introduction of fluorinated leucine analogs. (a) Ribbon model of coiled coil peptides with leucine residues highligted in yellow space filling mode. (b) CD spectrum of thermal denaturation curves of coiled peptides with either leucine (squares, filled 85 □M, open 35 □M) or trifluoroleucine (circles, filled 85 □M, open 35 □M).

a



b

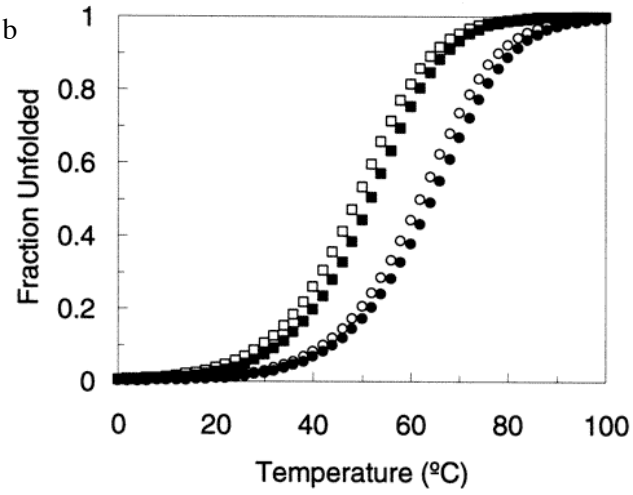
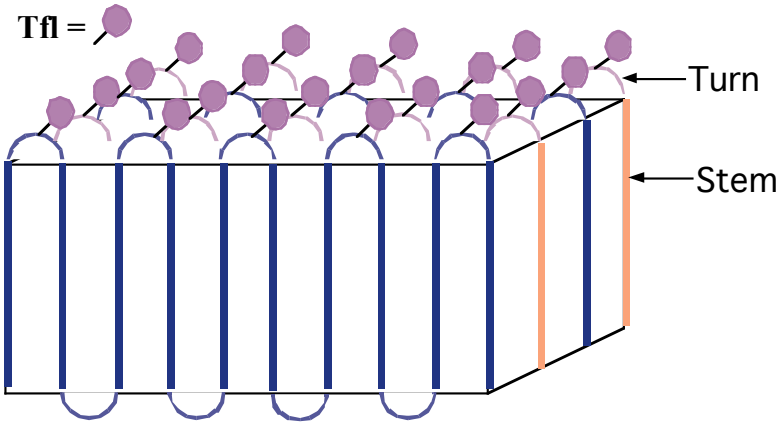


Figure 1.7. ß-lamellar structure exhibits surface properties defined by exposed amino acid. Diagram displaying ß-lamellar with leucine positions designated by balls on a stick at the interface.



{(GlyAla)$_3$GlyTfl}$_{12}$GlyAla

Contact Angle:

Leucine Polymer	17°
Trifluoroleucine	70°

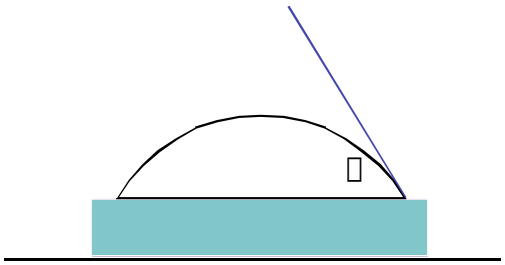
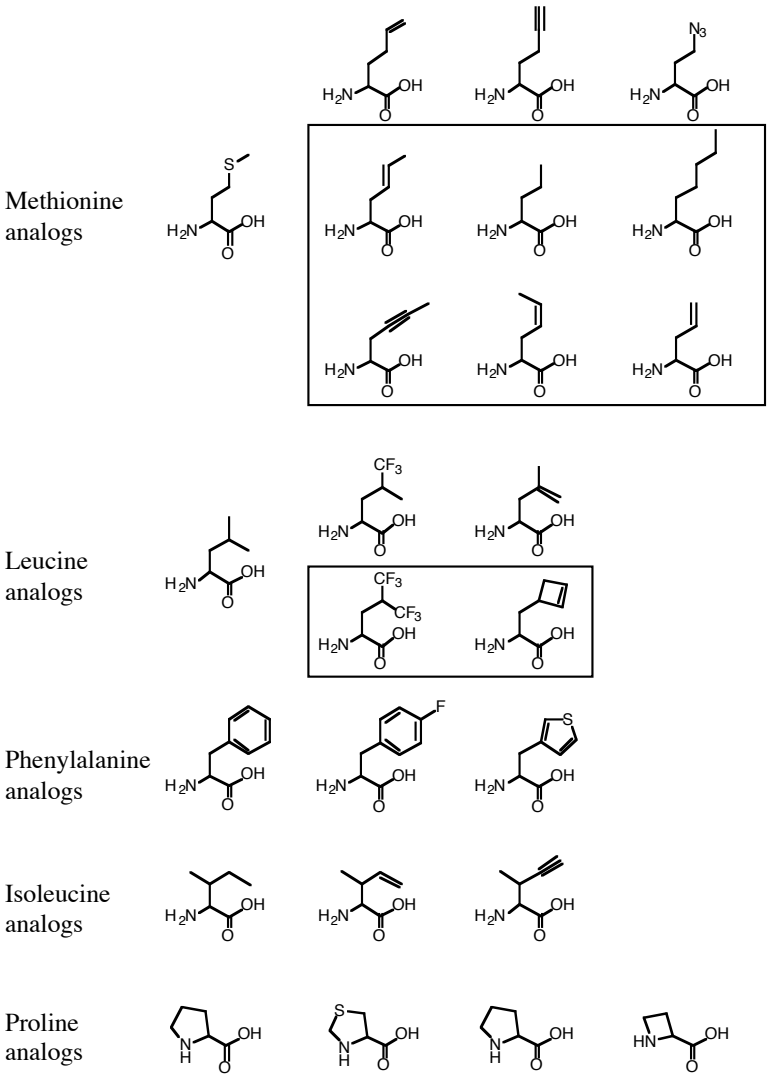


Figure 1.8. Subset of analogs incorporated *in vivo* via "media shift" method. Analogs in black are incorporated into auxotrophic strains with no further alteration of metabolism. Boxed residues required overexpression of given aaRS.



this end we developed a novel computationally designed mutant that was able to accept this analog (Chapter 3). In Chapter 4 we use *in vivo* protein production assays as well as *in vitro* kinetic assays to characterize the above two mutants plus an additional new mutant for their ability to tolerate a wide array of unnatural amino acids. Combined, these efforts resulted in the incorporation of a large number of unnatural amino acids, which differ substantially from phenylalanine in both size and electrostatic nature. These analogs also contain a large number of chemically interesting functionalities previously unknown within the context of proteins.

Multi-site incorporation of unnatural amino acids, afforded by the above method, is particularly useful in the construction of protein-based biomaterials [14]. To this end we produced an artificial extracellular matrix (aECM) protein, designed as a synthetic vascular graft material [48-50], which incorporates *para*-azidophenylalanine (pN_3Phe) [51-53] for the purpose of photochemical crosslinking (Chapter 5). The construct incorporates an endothelial cell-binding domain from fibronectin [54-56] and a structural motif derived from elastin, a natural structural protein within the vasculature [57]. This aECM construct was designed to avoid two problems commonly seen with synthetic vascular grafts, failure due to modulus mismatch and thrombosis resulting from the failure to form an endothelial cell lining [58-61]. Incorporation of pN_3Phe allows for crosslinking, via photolytic formation of the reactive nitrene, needed for the construct to form a cohesive vessel with the proper modulus able to withstand the pulsatile stress of the vasculature [62]. Photochemical crosslinking is advantageous because it avoids the used of chemical

crosslinkers which can cause difficulties with graft production and acceptance [63]. Photodecomposition of the arylazide also enables this protein to be used as a negative type photoresist [64]. Photopatterning spun aECM films armed with pN_3Phe provides a novel method of forming bioactive protein patterns, which is useful in a variety of biotechnologies [65-69]. Chapter 6 details photochemical patterning of our construct and the cellular patterns that develop in response to protein patterning.

Chemoselective ligations refer to a limited set of reactions that exhibit the ability to modify a specific chemical moiety in the presence of a large number of competing functionalities [4, 6, 11]. Our ability to introduce unnatural amino acids expands the number of selective chemistries that can be accessed for modification of biomolecules. Chapter 7 describes efforts directed towards development of Pd(0) cross coupling chemistry as a chemoselective chemistry [70-72]. Use of Pd(0) chemistry requires introduction of either an aryl halide, as phenylalanine analogs, or terminally unsaturated moieties, as either phenylalanine or methionine analogs [73-75]. Through the use of a model system and two protein systems we demonstrate that Pd(0) couplings satisfy the requirements for chemoselective ligations. These reactions proceed very well in water, do not generate any side reactions and are not affected by the natural amino acids, with the exception of cysteine in the case of Heck couplings. We demonstrate the use of these chemistries for the labeling of proteins, produced from *E. coli*, with epitope tags and fluorescent markers.

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