C h a p t e r I

INTRODUCTION

Traditional techniques of polymer synthesis produce macromolecules with statistical distributions of chain length, composition, stereochemistry, and sequence. Nature has evolved a complex system for polypeptide synthesis that gives essentially complete control of chain length and monomer sequence. Using the natural protein biosynthesis machinery to produce protein polymers provides not only a unique opportunity to study the effects of such molecular characteristics on material properties, but also the possibility of readily incorporating bioactive domains into protein-based materials.

Biomaterials produced through protein biosynthesis are limited, however, to the chemical functionality contained within the canonical set of 20 amino acids (Figure I-1). The incorporation of noncanonical amino acids makes possible the introduction of new functionality into proteins, creating the potential for novel material properties or interesting post-translational modifications. This thesis describes a series of investigations that i) expand the number of amino acids available for *in vivo* incorporation into proteins and ii) use the new physical and chemical properties provided by noncanonical amino acids to produce interesting materials.

The first step toward the goal of producing polypeptide polymers containing new amino acid functionality must be an understanding of how proteins are synthesized in nature.

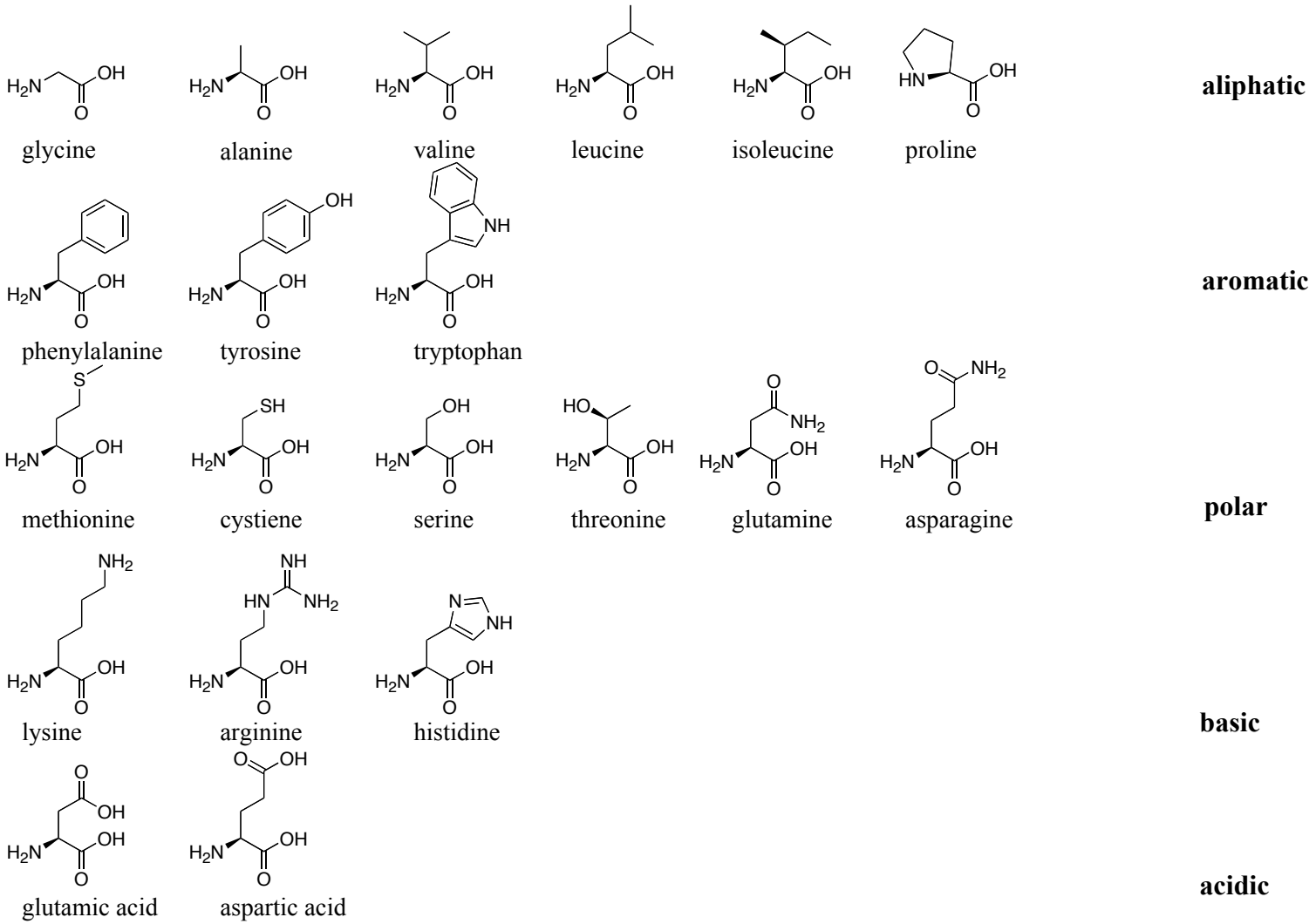

Figure I-1. The 20 canonical amino acids contain a variety of functional groups.

I.1 Protein Biosynthesis

Synthesis by the natural biosynthetic machinery involves transcription of DNA into mRNA and translation of mRNA codons into an amino acid sequence by tRNA (Figure I-2). Each tRNA contains a three-base anticodon that permits recognition of the codon(s) for the appropriate amino acid and, at the opposite end of the molecule, an adenine that serves as the attachment point for the amino acid. Amino acids are charged onto the tRNA by aminoacyl-tRNA synthetases (aaRSs); the aminoacyl-tRNAs are then transported to the ribosome by elongation factor Tu (EF-Tu). The ribosome, a multi-unit protein/RNA complex, guides the association of aminoacyl-tRNAs with their cognate mRNA codons, and a peptide bond is formed between the growing protein chain and the

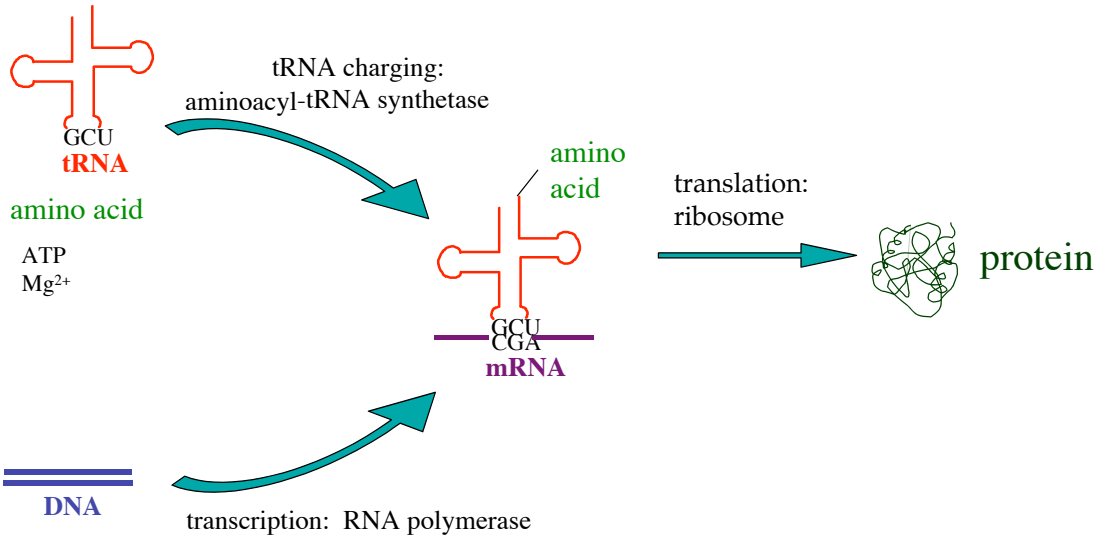

Figure I-2. During protein biosynthesis the genetic information encoded in DNA is transcribed into mRNA. The message is translated into an amino acid sequence in the ribosome where mRNA codons are "read" by the appropriate tRNA molecule that has been charged with its cognate amino acid by an aminoacyl-tRNA synthetase.

amino acid charged to the tRNA. The selectivity of this process for the appropriate amino acid is responsible for translational fidelity and is an important consideration in the attempt to incorporate noncanonical amino acids *in vivo*.

I.1.1 Fidelity of Protein Biosynthesis

There are a number of points during protein biosynthesis that provide an opportunity to assure that the correct amino acid is incorporated into the protein chain in response to a codon. The aaRSs recognize both the amino acid and one or more of its cognate tRNAs, EF-Tu binds the aminoacyl-tRNA to deliver it to the ribosome, and the ribosome itself recognizes the aminoacyl-tRNA.

There is considerable evidence that the aaRSs are largely responsible for the fidelity of protein synthesis. The ribosome discriminates between D- and L-α-amino acids [1-3] and rejects large aromatic analogues with certain geometries [4], but is otherwise relatively insensitive to amino acid structure. Indeed, in *in vitro* translation systems in which the amino acid is first attached to tRNA by chemical misacylation to bypass the aaRSs [5], the ribosome has been shown to accept and incorporate dozens of noncanonical amino acids into growing protein chains. Incorporated analogues include α-amino acids with side chains that are much larger and/or very chemically distinct from the canonical amino acids [6-8] as well as non-α-amino acids [9], including α-hydroxy acids [1, 10] and N-methyl amino acids [11, 12].

In the case of EF-Tu, it is known that the strength of binding of aminoacyl-tRNAs is optimized when the amino acid is attached to its cognate tRNA [13]. Yet, again, EF-Tu is promiscuous enough to transport to the ribosome a large number of misacylated tRNAs

produced both *in vitro* [14] and *in vivo* [15]. The majority of the burden of choosing the correct amino acid from the available intracellular pool rests with the aaRSs.

Each aaRS recognizes both an amino acid and its cognate tRNA(s). Although each aaRS has evolved to recognize a particular amino acid, common structural and functional domains have been identified (for review, see [16]). The aaRSs first catalyze the activation of the carboxyl group of the amino acid by reaction with adenosine triphosphate (ATP) to produce an aminoacyl adenylate. The activated ester of the aminoacyl adenylate reacts with the terminal hydroxyl group of tRNA to produce aminoacyl-tRNA. In some cases, the aaRS also catalyzes hydrolysis of improperly charged and/or activated amino acids.

Once the tRNA is aminoacylated by the aaRS, it is transported by EF-Tu to the ribosome where synthesis of the polypeptide chain is completed. Accurate decoding of genetic information is accomplished by the protein-mediated matching of amino acids with oligonucleotide sequence.

I.2 Protein Polymers

To the synthetic polymer chemist, an attractive feature of the protein biosynthesis machinery is its ability to precisely control the sequence of the growing polymer chain. In nature, such a fine degree of molecular control permits the production of proteins with invariable sequence and the ability to fold into well-defined, functional three-dimensional structures. Using the tools nature has developed provides the chemist with a way to design and produce protein-based polymers with controlled molecular architecture.

Early work in the Tirrell laboratory demonstrated the ability to introduce novel material properties through the precise control of polymer architecture afforded by the biosynthetic machinery. The rod-like polymer poly(γ-benzyl-L-glutamate) (PBLG) has been studied for its ability to form liquid crystalline phases; when produced with low polydispersity (~1.2) by standard synthetic techniques such as ring-opening polymerization of α-glutamic acid-N-carboxyanhydride, PBLG forms nematic liquid crystalline phases [17]. However, when PBLG is prepared through post-translational modification of bacterially produced poly(L-glutamic acid), a more ordered smectic phase is formed; mass spectroscopy of such samples show the chain population is of one uniform length [17].

Genetically templated synthesis also provides a straightforward method to include bioactive domains in the polymer structure. For example, the Tirrell laboratory has produced a family of proteins designed to mimic the natural extracellular matrix [18]. These modular constructs contain repeating blocks of protein sequence, one block derived from the structural protein elastin to impart appropriate mechanical properties and another block derived from known cell-adhesive sequences of the natural extracellular matrix protein fibronectin. Such proteins, when crosslinked by various methods, possess moduli within the range of native elastin [19,20] and adhere human umbilical vein endothelial cells (HUVEC) in a sequence-specific manner [21]. Studies involving these artificial extracellular matrix proteins (aECMs) will be the focus of Chapters 3 – 5 of this thesis.

Although *in vivo* synthesis of protein polymers has been shown to be a powerful technique, a clear limitation is the availability of only 20 monomers — the 20 canonical amino acids — with a limited range of functionality (Figure I-1). Expanding the number of monomers beyond these 20 would provide access to new chemical reactivity not available in wild type proteins.

I.3 Strategies for the Incorporation of Noncanonical Amino Acids

The protein biosynthetic machinery makes mistakes; error frequencies for misreading a codon and incorporating the incorrect canonical amino acid are ~10^{-4} [22]. It has also long been recognized that certain noncanonical amino acids, e.g., selenomethionine [23], can infiltrate the biosynthetic machinery and be incorporated into proteins in place of a structurally similar amino acid. Work in several laboratories has exploited this promiscuity to incorporate amino acids with a variety of functional groups not contained within the canonical set [24-32].

To further expand the set of functional groups available to protein engineers, several groups have been developing techniques to incorporate noncanonical amino acids, both in bacterial and mammalian cells as well as in cell-free *in vitro* translation systems. Figure I-3 schematically illustrates the major strategies being applied for the incorporation of noncanonical amino acids into proteins *in vitro* and *in vivo*.

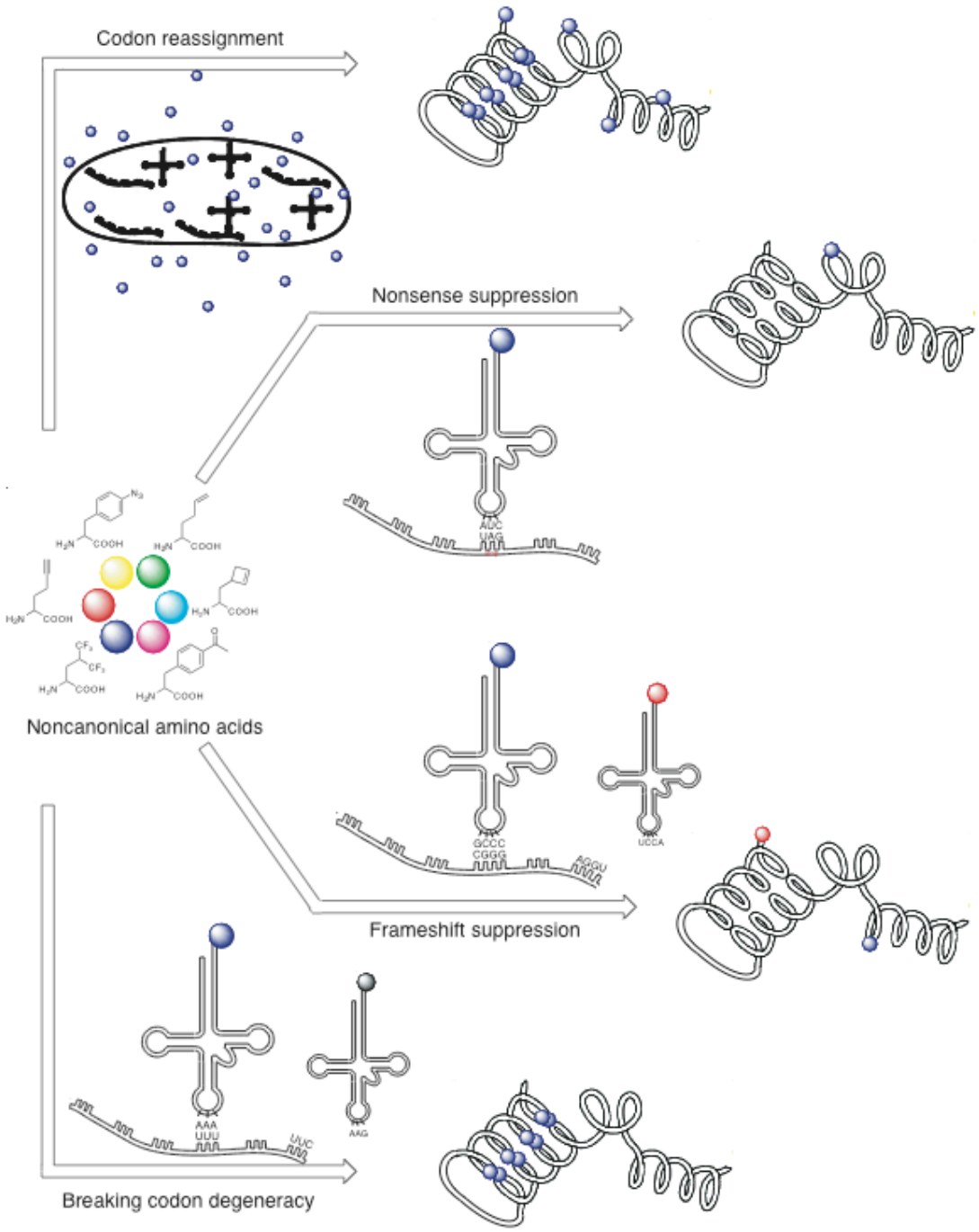

Figure I-3. Schematic representation of the various strategies for incorporating noncanonical amino acids into proteins. This figure appeared in [15].

I.3.1 In vitro *incorporation*

In the late 1980s Chamberlin [33] and Schultz [34] introduced an *in vitro* strategy for incorporation of noncanonical amino acids through nonsense suppression by chemically misacylated tRNA. Many groups have since used this technology for site-specific incorporation to study protein structure and function.

The nonsense suppression strategy uses translational read-through by a chemically misacylated suppressor tRNA to incorporate noncanonical amino acids site-specifically in response to a nonsense codon, usually the amber stop codon (TAG). Because of limitations in suppressor efficiencies, protein yields are generally low (1 – 10 μg/mL) [35], but the precise control of the placement of the noncanonical analogue makes this a powerful tool for studies of protein structure and function.

For example, Pollitt and coworkers have employed site-specific incorporation *in vitro* to cage an aspartic acid side chain of a protein ($p21^{ras}$) as its *o*-nitrobenzyl ester, allowing them to photochemically control its interaction with a protein partner (p120-GAP) [36]. Koh and coworkers have investigated the role of specific backbone amide linkages in T4 lysozyme through substitution with ester bonds by incorporation of α-hydroxy acids [10].

Site-specific incorporation has also been accomplished using codons other than stop codons. Frameshift suppression of four-base codons has been used by Sisido and coworkers to incorporate a fluorophore-quencher pair at selected positions in strepavidin

[37], and use of unnatural nucleosides *iso*-C and *iso*-G enabled the Hecht group to incorporate iodotyrosine in response to the "65th codon" [38].

I.3.2 In vivo *incorporation*

I.3.2.1 Residue-specific incorporation (codon reassignment)

By reassigning all of the codons for a particular amino acid to a close structural analogue, the noncanonical analogue can be incorporated at multiple sites throughout the protein. Using this technique, one can significantly change the physical properties of a protein [28, 39, 40] or provide for multiple reactive sites for chemical modification, such as labeling [25, 41] or crosslinking (Chapter 4).

Often codon reassignment is accomplished simply by starving an auxotrophic *Escherichia coli* (*E. coli*) strain for a canonical amino acid in medium supplemented with a close structural analogue. Tang and coworkers [40] employed this strategy to replace up to 92% of the leucine resides in the leucine-zipper protein A1 with trifluoroisoleucine. The fluorinated leucine zippers displayed a 13°C increase in melting temperature (T_m) as well as increased resistance to chemical denaturation. Kiick and coworkers [25] also used this method to incorporate the noncanonical amino acid azidohomoalanine into a target protein, murine dihydrofolate reductase (mDHFR), which they then chemoselectively labeled through Staudinger ligation with a phosphine bearing an antigenic peptide.

Access to a wider range of noncanonical amino acids for residue-specific incorporation is provided through alterations of the wild-type biosynthetic machinery. Overexpression of the appropriate synthetase can improve the incorporation of poorly activated analogues [39,42]. A more general strategy involves mutating the aaRS to alter its substrate specificity at either the synthetic [24,26,43] or hydrolytic [44,45] active site. For example, Doring and coworkers demonstrated replacement of valine by the noncanonical amino acid aminobutyrate in a strain harboring valyl-tRNA synthetase (ValRS) with multiple mutations in the editing site [45].

A previously characterized mutant *E. coli* phenylalanyl-tRNA synthetase (PheRS) [24,46], with an enlarged active site resulting from the mutation of residue 294 from alanine to glycine, accepts a wide variety of phenylalanine analogues not incorporated by wild-type *E. coli* hosts, including the photoreactive amino acid *para*-azidophenylalanine [26]. Photocrosslinking of artificial proteins through this noncanonical amino acid is described in Chapter 4 of this thesis.

I.3.2.2 Site-specific incorporation

Several research groups have developed technologies to site-specifically incorporate noncanonical amino acids *in vivo*. Lester and coworkers first demonstrated site-specific incorporation *in vivo* through injection of chemically misacylated tRNA into *Xenopus* oocytes [14] and have more recently expanded this strategy to mammalian cells [47].

Furter [48] developed a successful system for site-specific incorporation in *E. coli* by importing a yeast suppressor tRNA and yeast aminoacyl-tRNA synthetase to generate

a "21st pair." Because the native *E. coli* $tRNA^{Phe}$/PheRS pair was incapable of activating *p*-fluorophenylalanine, this noncanonical analogue was selectively incorporated by the heterologous 21st pair in response to amber stop codons.

Schultz and coworkers have expanded and improved upon this strategy. Through directed evolution of aaRSs that show high specificity toward a noncanoncial amino acid [49], they have created *E. coli* hosts with 21st pairs incorporating *p*-acetylphenylalanine [50] and benzophenone [51], among others. They have also generated an organism with a novel 21st amino acid by inserting an aaRS evolved to incorporate *p*-aminophenylalanine and the biosynthetic genes for the amino acid into an *E. coli* host [52]. Use of orthogonal 21st pairs has recently been expanded to *Saccharomyces. cerevisiae* [53] and Chinese hamster ovary cells [54].

I.3.2.3 Multiple site-specific incorporation

Kwon and coworkers have described a system that takes advantage of the degeneracy of the genetic code to "reassign" the phenylalanine wobble codon UUU to the noncanonical amino acid 2-napthylalanine [55]. This technique is complementary to other missense strategies such as nonsense and frameshift suppression, but offers the unique ability to efficiently incorporate a noncanonical amino acid at multiple pre-selected sites, e.g., throughout a single protein domain.

Using the strategies described in Section I.3.2, to date the Tirrell laboratory has successfully incorporated the set of amino acids shown in Figure I-4 into proteins *in vivo*.

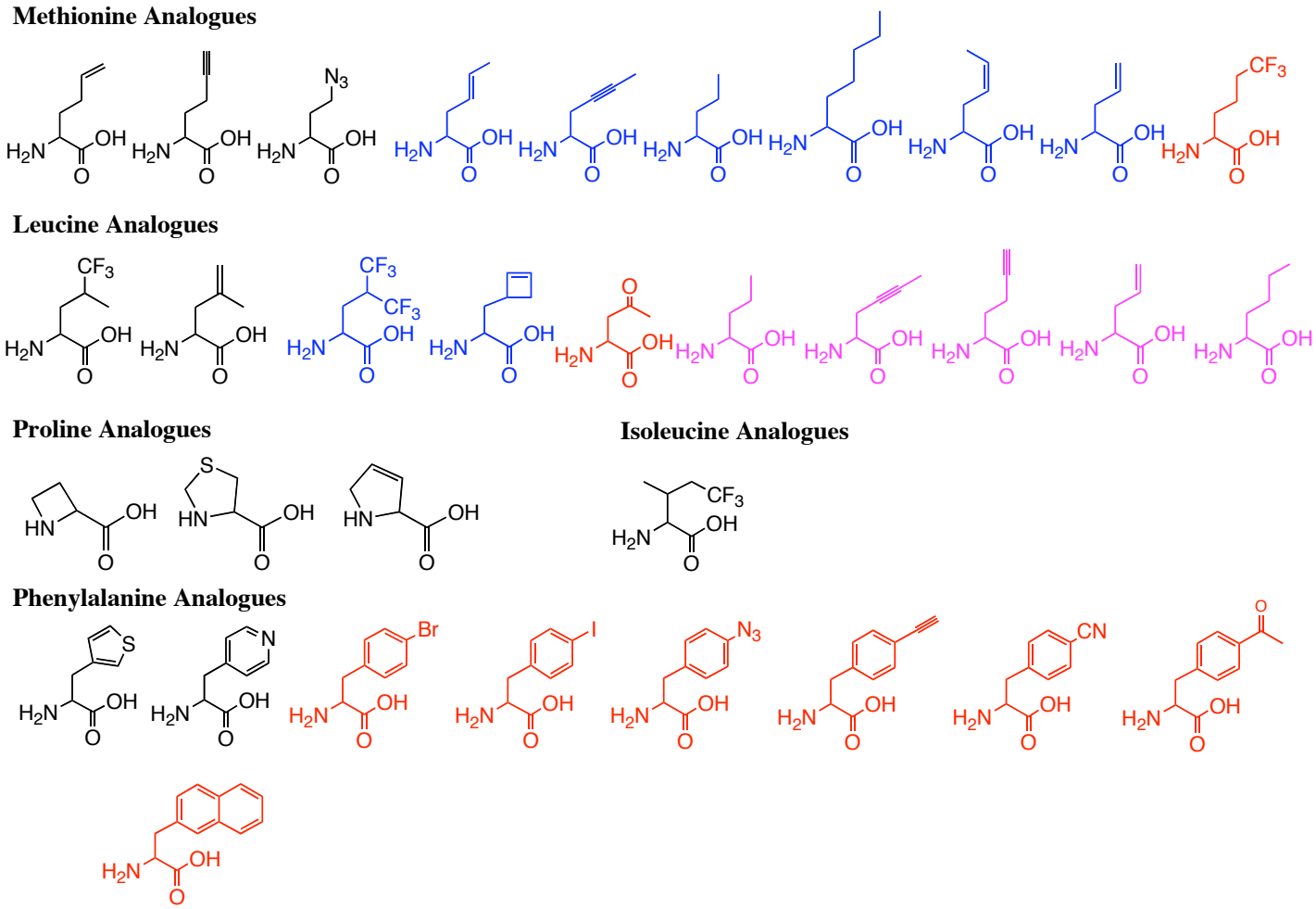

Figure I-4. Noncanonical amino acids incorporated into proteins *in vivo* by the Tirrell laboratory. Amino acids in black are accepted by the wild type *E. coli* biosynthetic machinery; those in blue require overexpression of the wild type aaRS; those in red require an active site mutant aaRS; and those in purple require an editing site mutant aaRS.

I.4 Thesis Objective

The objective of this thesis work was to expand upon the set of amino acids available for incorporation into proteins *in vivo* and to explore applications of the novel chemistries and physical properties provided by the new analogues.

Chapter 2 describes the incorporation of new, unsaturated analogues of isoleucine, the alkene 2-amino-3-methyl-4-pentenoic acid (E-Ile) and the alkyne 2-amino-3-methyl-4-pentynoic acid (Y-Ile), by the wild type *E. coli* biosynthetic apparatus. The IleRS was found to be sensitive to sidechain stereochemistry in the case of the alkene analogue; the translational activity of the pairs of enantiomers (SS, RR-E-Ile and SR, RS-E-Ile) are markedly different. We conclude that although SS-E-Ile is a good substrate for the IleRS, SR-E-Ile is not incorporated into proteins by wild type *E. coli* translational machinery. At least one stereoisomer of the alkyne analogue of Ile is also translationally active, although the level of stereochemical purity of the amino acid samples was not sufficient to determine whether a similar stereochemical discrimination existed for Y-Ile.

Chapter 3 focuses on the incorporation of a fluorinated noncanonical amino acid, 5,5,5-trifluoroisoleucine (5TFI), into artificial extracellular matrix proteins. The fluorinated aECMs displayed altered phase behavior and were more resistant to degradation by the physiologically relevant protease elastase, yet retained the ability to adhere endothelial cells in a sequence specific manner.

Chapter 4 describes the incorporation of the photoreactive noncanonical analogue *p*-azidophenylalanine (*p*N_3Phe) into artificial extracellular matrix proteins using an *E. coli* host expressing a mutant PheRS (A294G) in which the active site has been expanded

to accommodate larger amino acids. Films of the azide-containing aECMs were crosslinked upon short (~30 s) exposure to ultraviolet radiation at 365 nm. Using simple patterned masks, we demonstrated the ability to pattern protein films by only exposing certain regions. When protein patterns are produced on a non-adhesive background, endothelial cells selectively adhere to the protein regions to create stable cell patterns.

Chapter 5 describes progress toward the cloning of a new aECM construct containing a very robust cell-adhesive domain as well as regular phenylalanine sites for incorporation of *p*N_3Phe.

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