Chapter 4

Future Directions for the Discovery of New Methionyl-tRNA Synthetase Activities

Ligand Recognition by Methionyl-tRNA Synthetase and Its Mutants

The methionine (Met) side chain is commonly accepted to be hydrophobic. However, unlike the related valyl-, isoleucyl- and leucyl-tRNA synthetases, the amino acid binding pocket of the methionyl-tRNA synthetase (MetRS) contains a series of polar residues forming an intricate network of hydrogen bonds. (Figures 3.1.b; Figure C.1) Upon Met binding, these polar groups recognize the ligand side chain by donating hydrogen bonds to the Sδ atom on Met. It is foreseeable that in the absence of these polar contacts ligand selectivity would be compromised and other hydrophobic moieties in the environment might compete with Met for the binding site. In addition, the MetRS binding pocket experiences a large conformational change upon ligand binding. To enable this conformational change, the W253 residue, which rotates more than 90° around χ1 and forms one side of the binding pocket, has to be labile. (Figure 2.16.a) Because of this, small incompatibilities that arise when binding a non-cognate ligand might be better tolerated by MetRS than other similar synthetases, like isoleucyl- or valyl-tRNA synthetase. Indeed, in a computational study of the fidelity of MetRS to ligand binding, Datta et al. identified leucine (Leu) as the primary competitor of Met [1]. (Figure 4.1) Thus, having ligand-specific hydrogen bonding interactions likely allows MetRS to correctly identify Met among other hydrophobic amino acids.

Although hydrogen bonds can help in ligand recognition, hydrogen bonds to organic sulfur atoms are relatively weak and hydrogen bonds are not very prevalent for methionine residues in proteins [2, 3]. Therefore, while the availability of hydrogen bond donors in the binding site will improve discrimination against hydrophobic amino acids, it may also create new competitors by allowing polar amino acids to bind MetRS. Although the interaction of any polar competitor with the MetRS binding pocket will be suboptimal, the competitor may compensate for that by forming strong hydrogen bonds with the donor groups in the binding site. The amino acids glutamic acid (Glu) and glutamine (Gln) were identified by Datta et al. as the best competitors for the MetRS binding site after Met and Leu [1]. Interestingly, when methionine-auxotrophic *E. coli* with elevated

levels of the wild-type MetRS is induced to express protein in a methionine-deficient medium, expression of protein is observed. (Figure B.1) Mass spectrometry analysis of tryptic fragments from the expressed protein indicate the replacement of Met by Glu. (Figure B.3, first panel) This observation supports the notion that MetRS needs a fine balance between hydrophobic and polar elements in the binding site to be able to recognize the Met side chain, which is hydrophobic but also is a weak hydrogen bond acceptor.

In our studies, we aimed to discover mutations in the MetRS binding site that permit the activation and the subsequent charging of the noncanonical amino acid azidonorleucine (Anl) onto $tRNA^{Met}$. By screening a MetRS saturation mutagenesis library, we identified a diverse set of mutants that allow azidonorleucine (Anl) incorporation *in vivo*. Quantum mechanical (QM) calculations on the azide group indicate a high extent of charge separation between the azide nitrogens, making the central nitrogen atom electron poor (δ = +0.5) with respect to the terminal and base nitrogen atoms (δ = -0.3 and -0.5, respectively). Thus, mutations that recognize the polar character of the azide group were anticipated from the screens *in vivo*. However, the results of the screens revealed hydrophobic substitutions for Y260 and H301, the two sites best positioned for polar interactions with Anl. While the experimentally obtained MetRS mutants preferred non-polar side chains, the computational models predicted that many mutants in the saturation mutagenesis library could hydrogen bond with the azide group. If hydrogen bonding interactions are available for the ligand, it is puzzling why such interactions are not utilized.

A survey of crystal structures of organic azides concluded that hydrogen bonds to organic azides are weak based on their frequency and the donor-acceptor distance [4]. It is possible, then, that the same problem is encountered with Anl, as with MetRS and Met. Placement of polar groups into the binding site may promote polar amino acids competing for this site. Unlike in the wild-type MetRS, the screen might be choosing hydrophobic residues compatible with binding Anl to avoid such a competition. The L13G mutant of MetRS, which displays good activation kinetics for Anl, is an example of a mutant that suffers

from a competition with polar amino acids. In the absence of Met and Anl, the L13G mutant allows the incorporation of glutamine (Gln) or lysine (Lys) into Met sites in proteins, based on the MALDI-MS analysis of tryptic fragments. (Figure 2.10) The screening strategy we employ for selecting MetRS mutants active toward Anl is also sensitive toward the presence of any competitors. Met is a strong competitor against Anl for the MetRS-L13G binding site and the cell-surface fluorescence labeling drops sharply in the presence of Met for cells equipped with this mutant. (Figure 2.11) Gln and/or Lys are weaker competitors than Met and their incorporation through MetRS-L13G allows them to replace Anl in about 50% of the Met sites in the presence of 0.3 mM Anl, and 0.27 mM Gln and Lys. This likely is responsible for the lower cell-surface fluorescence levels achieved through this mutant at 0.3 mM Anl (Figure 2.8) when compared with other MetRS mutants that have similar Anl activation characteristics, such as the NLL and SLL mutants (Table 2.3), and explains why this mutant was not encountered in the screens performed.

Selected MetRS Mutants May Display Broad Activities

The selection of hydrophobic side chains in the MetRS binding site opens the possibility for other aliphatic amino acids with largely hydrophobic side chains to be incorporated by the selected enzymes. The SLL mutant was previously identified by Yoo and Tirrell in an independent screen of a MetRS library for the incorporation of 6,6,6-trifluoronorleucine (Tfn) [5]. Ethionylnorleucine (Enl), which bears the alkyne functionality and can be ligated to terminal azides through a Cu(I)-catalyzed [3+2] azide-alkyne ligation, is another ligand of interest for MetRS. When a series of MetRS mutants previously characterized with Anl (Figure 2.9) were tested for protein expression in the presence of Enl and absence of Met, protein expression was achieved through all MetRS mutants tested, strongly suggesting the incorporation of Enl into proteins. (Figure 4.2.)

The screen by Yoo and Tirrell employed both negative (against incorporation of 20 canonical amino acids) and positive (for incorporation of Tfn) screening strategies to reach the MetRS-SLL mutant that allows Tfn

incorporation. Screens we have carried out for Anl incorporation in the presence of 20 canonical amino acids have returned the NLL and PLL mutants for incorporating Anl, while discriminating against canonical amino acids. (Figure 2.7; Table 2.2) These results suggest that Y260L-H301L mutants of MetRS that also carry a small and/or polar residue at position 13 (XLL) may be less sensitive to competition by Met and other canonical amino acids. These mutants may, therefore, be good candidates for exploring new noncanonical amino acids for incorporation into proteins *in vivo*.

Creating a More Hydrophobic MetRS Binding Pocket

MetRS mutants of the XLL-type present a more hydrophobic binding pocket than wild-type MetRS, but they still carry a water molecule deep in the binding pocket. In the MetRS-SLL crystal structure, this water molecule interacts with the terminal azide nitrogen of Anl through a hydrogen bond [6] (Figures 3.1.c) and its presence in the binding site is detected in various ligand-bound and ligand-free structures of MetRS [6-9]. The presence of a hydrogen-bond donor deep in the binding pocket may allow better recognition of ligands that carry a terminal hydrogen-bond acceptor. Nζ-acetyllysine (NaK) may be such a ligand. This amino acid is produced *in vivo* through the enzymatic acetylation of lysine, and is implicated in histone reorganization [10]. The terminal amide oxygen might enable this ligand to be efficiently recognized by the XLL-type mutants. However, the presence of this water molecule may interfere with the activation of amino acids such as 2-aminooctanoic acid (Aoc), which do not have terminal polar groups. In fact, the MetRS-SLL mutant allows only very limited levels of protein expression in the presence of this ligand [11]. For efficient incorporation of such amino acids, it may be necessary to remodel the binding pocket to exclude this water molecule.

In the MetRS binding pocket, the water molecule is held tightly by a hydrogen bond from the side chain of residue T10, and a hydrogen bond to the backbone carbonyl group of F292. (Figure 4.3) Although the second hydrogen of the water molecule forms a hydrogen bond with the ligand in the Anl-bound

MetRS-SLL crystal structure, in the wild-type enzyme this hydrogen bond is formed with the Oη atom on the Y260 side chain. (Figure 3.1.b.) Because the Y260 Oη atom also engages in a hydrogen bond with the ligand (Met) in the wild-type MetRS crystal structure, it was suggested that this water molecule was important for enzyme function [8]. However, because many different types of mutations are permitted at position 260, we believe that the removal of the water molecule through mutations may also be permitted. In addition to its hydrogen bond to the water, the T10 residue is stabilized by a hydrogen bond from the H291 side chain. It may be possible to engineer positions 10 and 291, together with the nearby position 260, and determine if it is possible to replace this hydrophilic region in the binding site with hydrophobic residues. Such a modification may create a MetRS binding pocket with more hydrophobic character, and enable better incorporation of long-chain hydrophobic residues such as Aoc.

Proteomic Applications of MetRS-NLL

Azidohomoalanine (Aha) is an azide-bearing amino acid, readily incorporated into proteins through the wild-type MetRS in bacterial and eukaryotic cells [12, 13]. Taking advantage of this and the bio-orthogonal nature of the azide group, Dieterich and co-workers showed that proteins synthesized in cells during an Aha ‘pulse’ can be tagged with the azide group. This enabled the researchers to selectively enrich proteins synthesized in neurons in response to stimuli, and analyze their composition [13]. Called bio-orthogonal noncanonical amino acid tagging (BONCAT), this method promises to significantly decrease sample complexity during proteomic analyses by restricting the sample composition to proteins synthesized at events of interest in a cell’s life.

However, because Aha is taken up by a large variety of cells, this method has limited applications to situations where the response of a single organism, or cell type is to be investigated in a mixture of cells. In investigations of neurons in the presence of glia, or of microbes infecting a eukaryotic host, where proteins from the subject make up a small fraction of the total protein mass, gathering

useful proteomic data from the subject can be very challenging. In these cases, it is not only necessary to restrict metabolic labeling temporally, but also to a specific cell type in the culture. This can be accomplished by giving the subject the ability to incorporate an azide-bearing amino acid like Anl that is not incorporated by the wild-type protein synthesis machinery. By equipping only the subject with a mutant MetRS (MetRS-NLL), metabolic labeling can be directed to only the cells of interest. Other cells in the culture lacking an Anl-active mutant MetRS will be inert to this amino acid. Proteomic responses from the subject can then be examined through the use of a variety of reactive probes for isolation or visualization. Ngo, Champion and co-workers have have demonstrated the utility of this strategy in bacterial co-cultures, as well as in co-cultures of bacteria and murine alveolar macrophages [14]. By infecting macrophages with *E. coli* equipped with MetRS-NLL in the presence of Anl, the researchers were successful in restricting metabolic labeling to proteins in the bacteria, almost exclusively.

The success of this study establishes the use of mutant AARSs to direct metabolic labeling. This is a brand new role for the AARS. The above concept is applicable to other reactive amino acids rejected by the wild-type metabolism, such as Enl. It is easy to envision simultaneous labeling of interacting cells that make up an organism, through the use of multiple AARS mutants and ligands. Such studies may better expose intricate connections in complex cell communities. Therefore, the current effort proves a starting point for many future biological and structure-based design studies.

Figure 4.1.

Structures of natural and noncanonical methionine analogs.

The chemical structure of methionine is shown with other natural amino acids that may be substrates for the wild-type or mutant MetRS. Structures for several noncanonical amino acids that are or may be substrates for MetRS mutants are also displayed.

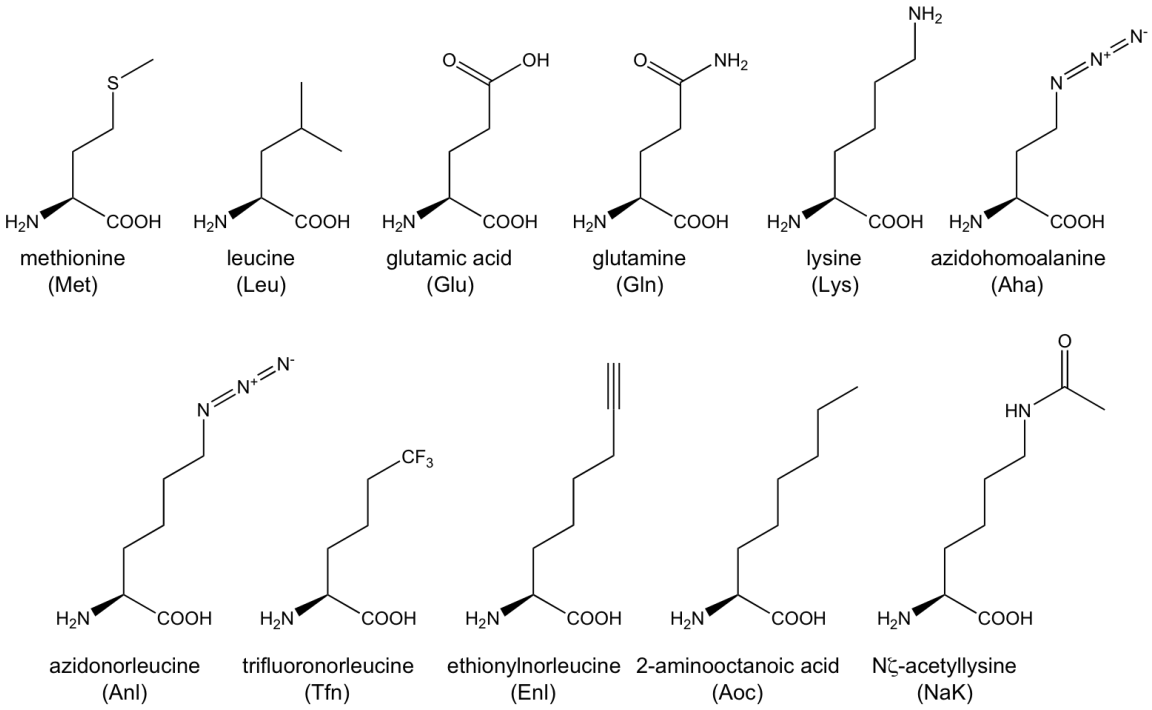


Figure 4.2.

Expression of DHFR in the presence of Enl in cells bearing various MetRS mutants.

DHFR was expressed in M15MA[pREP4/pAJL-61] cells encoding the MetRS mutants isolated from screens carried out at 0.3 mM (blue) and 1.0 mM Anl (green), as well as the L13G mutant and wild-type MetRS. Expression was done in M9+19aa media supplemented with 2.0 mM *dl*-Enl (gift from John T. Ngo; labeled "+Enl"), 40 mg/L Met ("+Met"), or no 20th amino acid ("19aa"). An aliquot taken before induction is labeled "pre-ind." SDS-PAGE analysis of whole-cell lysates show DHFR expression in the presence of Enl for all MetRS mutants.

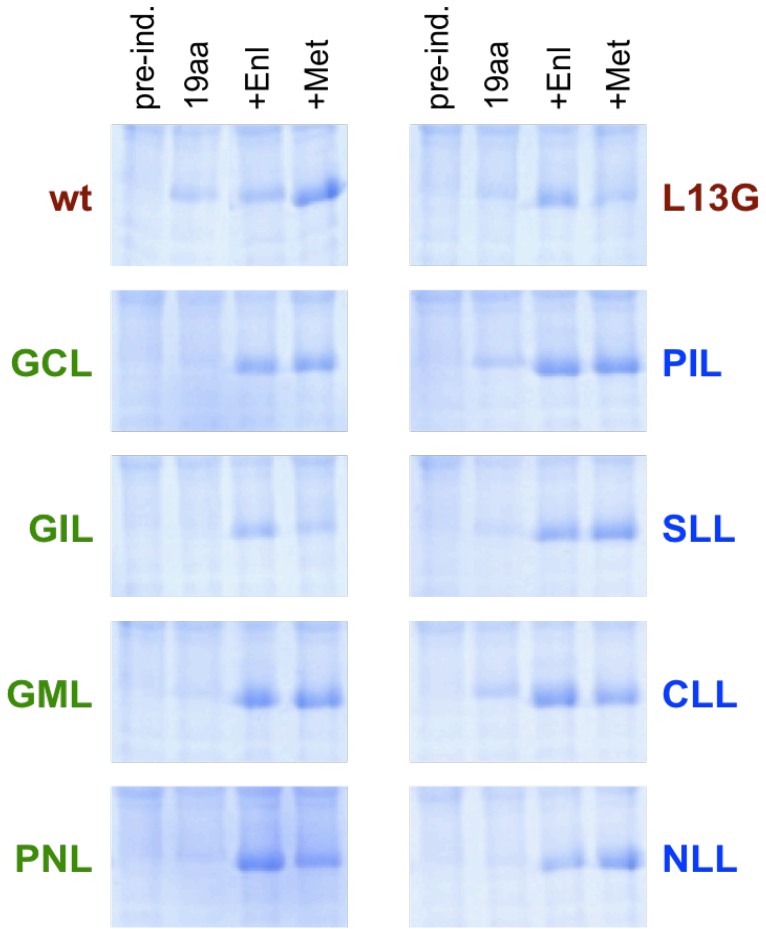
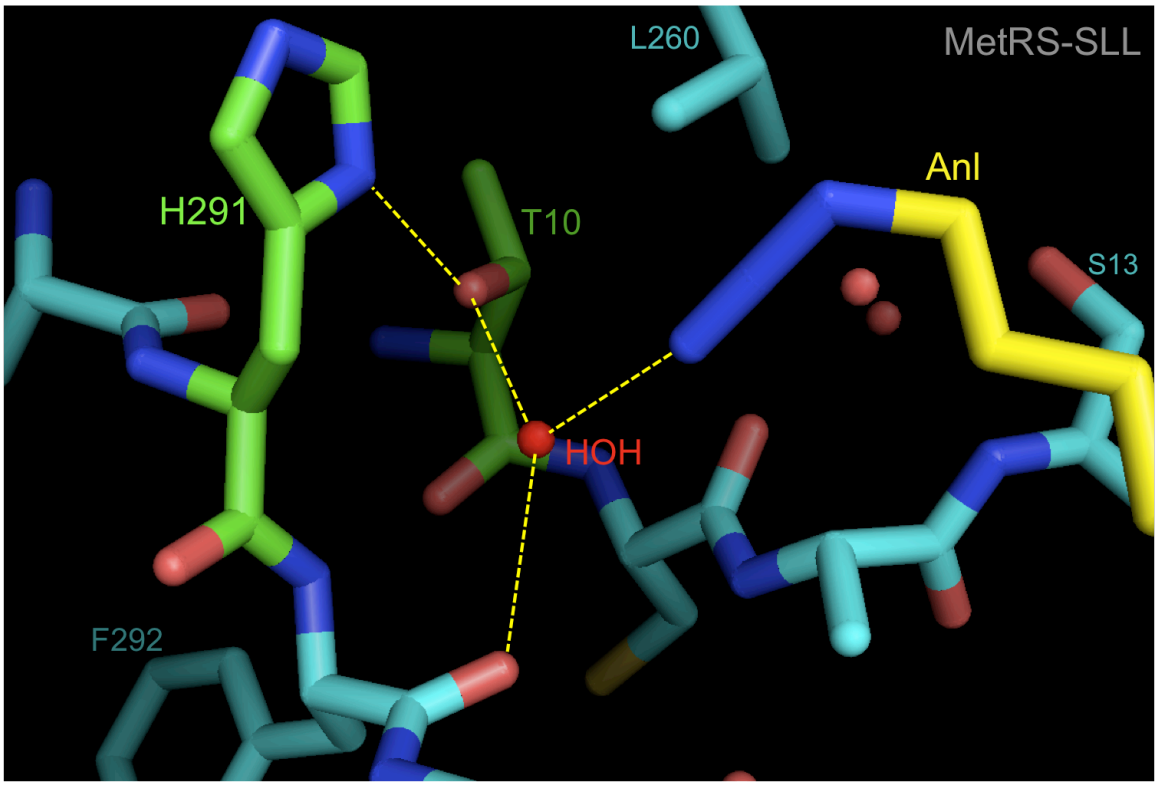


Figure 4.3.

Interactions of a conserved water molecule in the MetRS binding site.

The interactions of the conserved water molecule with the MetRS-SLL binding site and the ligand Anl are shown. The water molecule is labeled “HOH” and dashed lines mark important hydrogen bonds between the ligand and residues in the binding site. The residues in the binding site that facilitate the association of the water molecule are shown in green, and the ligand in yellow. This representation was prepared using PyMol, based on coordinates from [6].



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