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Development of a Site-Selective Protein Immobilization Methodology Utilizing Unnatural Amino Acids

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A thesis submitted in partial fulfillment of the requirement

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by

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Abstract

Proteins are nature's catalysts and have evolved over millennia to be highly selective and efficient. As a result, many have sought to incorporate proteins into artificial systems to varying degrees of success. Immobilization of proteins onto solid supports can increase the stability of proteins in conditions that would normally induce denaturation but immobilization strategies can present their own challenges by using reactions that lack selectivity and can potentially disrupt protein function themselves. This work develops a new methodology for protein immobilization that uses an unnatural amino acid site-selectively incorporated into a protein as the functional handle for immobilization. As a model system, Green Fluorescent Protein was immobilized in this manner and found to retain its functional activity in conditions which would normally cause the protein to denature. Through the development of this methodology, it was found that immobilization efficiency is affected by the site where the functional handle is inserted, in addition to the accessibility and reactivity of the solid support. Finally, progress was made towards determining whether this methodology can be successfully incorporated into a system which utilizes microwave irradiation.

Chapter 1: Introduction

1. A. Proteins in Non-Physiological Environments

Incredibly diverse and ubiquitous, proteins are the major workhorses of the cell, and for years researchers have sought to utilize their catalytic capabilities in a laboratory setting. Over millennia though, proteins have evolved to operate in specific physiological environments and are very sensitive to environmental conditions. Thus, they tend to denature in nonpolar solvents, high or low pH environments, and elevated temperatures, all of which are unfortunately hallmarks of synthetic organic chemistry and the environments typically encountered in non-biological settings.[1] Indeed, most organic reactions involve reagents and intermediates that are insoluble in aqueous environments and thus necessitate the use of organic solvents, further complicating the potential use of enzymes (catalytic proteins) in the catalysis of these reactions. Therefore, there has been a large push in the field of chemical biology to find efficient ways to modulate enzymes to increase their functionality beyond what is observed in nature. In a related vein, researchers are also very interested in methods which can more efficiently integrate proteins into artificial systems while preserving their integrity and catalytic prowess.[2] Processes that allow for the fast, efficient, and reliable integration of a diverse population of modified enzymes into artificial systems will surely revolutionize chemical synthesis and chemical biology in the years to come by allowing for optimized synthetic pathways that can tightly control stereochemistry and also more efficiently generate large chemical libraries of small molecules for investigation with minimal waste and byproduct production.

1. B. Incorporation of Unnatural Amino Acids

With the exception of a few organisms, almost all of life relies on only the twenty canonical amino acids to synthesize proteins. The amount of functional diversity that is achieved through various combinations of these twenty compounds is truly amazing, but the existence of necessary enzyme cofactors and post-translational modifications clearly indicate that sometimes additional chemical diversity is necessary for certain cellular functions.[3] Along this line of thinking, it is interesting to consider which common chemical functionalities are completely absent from the twenty canonical amino acids. For example, no natural amino acid contains a halogen or common functional groups such as
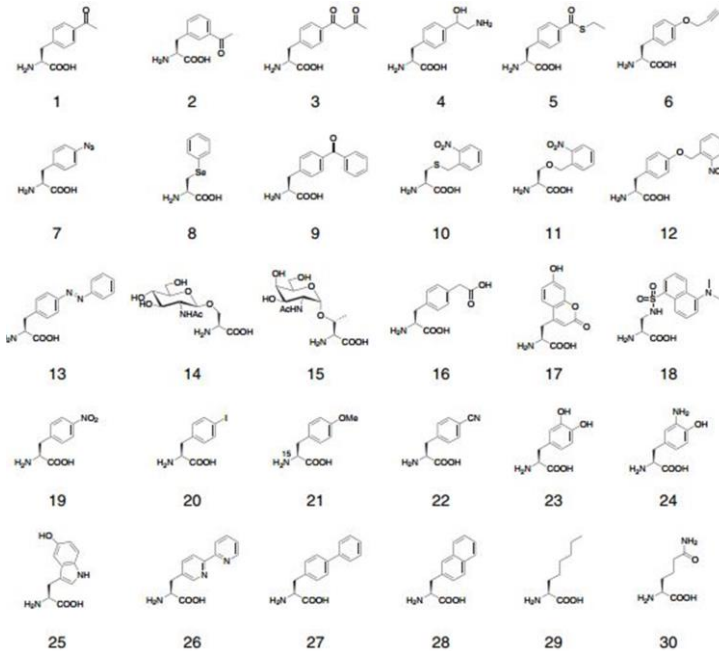
Figure 1.1. Various unnatural amino acids that have been added to the genetic codes of *E. coli*, yeast, or mammalian cells. Adapted from *Annu. Rev. Biophys. Biomol. Struct*. **2006**, *35*, 225–249.[4]

a ketone, alkyne or an azide. To explore the possibilities of adding these functionalities to nature, researchers have synthesized entire libraries of unnatural amino acids (UAAs). (See **Fig. 1.1**) These compounds share the same backbone as the canonical amino acids but have vast differences in their side chains. Theoretically, the side chain of an amino acid could

be synthesized to contain almost any common organic functional group imaginable to generate unique amino acids with highly diverse chemical properties.

Once these unnatural amino acids have been synthesized however, the main obstacle is actually producing proteins that have an unnatural amino acid inserted into their primary sequence. During the normal biological process of translation, three nucleotide long codons on an mRNA transcript pair with aminoacyl-tRNAs that contain a complementary sequence inside of the ribosome.[1] These aminoacyl-tRNAs are produced when tRNAs are ligated (charged) to a specific amino acid by an aminoacyl tRNA synthetase (aaRS).[1] Thus, tRNAs function to deliver amino acids to a ribosome and translate a nucleotide sequence into a peptide sequence which then can fold into functional protein.[1] Translation begins at the start codon (AUG) which also codes for methionine and ends when one of the stop codons (UAG, UAA, UGA), which do not code for any amino acid, is reached.[1] Stop codons bind to release factors instead of aminoacyl-tRNAs and cause the dissociation of ribosome subunits, ending translation.[1] As this system has evolved over millennia to very selectively incorporate amino acids that are recognized by natural aminoacyl tRNA synthetases, early efforts to incorporate unnatural amino acids focused on non-biological systems. Methods such as solid-phase peptide synthesis, and chemically aminoacylating a nonsense suppressor tRNA have been demonstrated in the literature but are largely inefficient.[3,4] Solid-phase peptide synthesis involves the step-wise synthesis of a peptide through adding protected amino acids one at a time onto a solid support then de-protecting them for the next cycle.[4] This method can allow for a large number of modifications but is largely inefficient for producing anything other than short peptides or small proteins as yields and purity decrease with each cycle.[4] Chemically aminoacylating

a nonsense suppressor tRNA involves attaching an unnatural amino acid to a tRNA which is complementary to a stop codon and thus can suppress the end of translation by instead inserting an unnatural amino acid.[3] This method can allow for the incorporation of an unnatural amino acid into any length of protein but is chemically complex and cell-free translation yields are low.[4] A more viable method involves modifying a cell's existing protein machinery to incorporate unnatural amino acids. This method can allow for the efficient production of any length of protein and is equivalent to an expansion of an organism's genetic code to include an unnatural amino acid. This methodology has been used to incorporate >50 unnatural amino acids into the proteins of bacterial and mammalian cells as
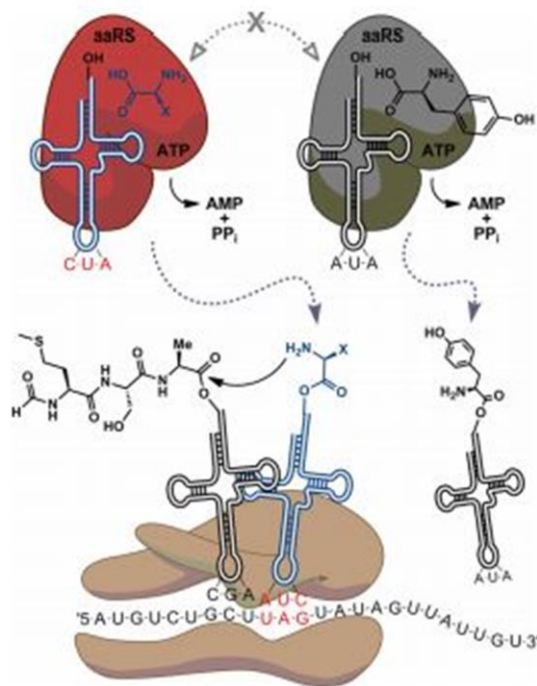
Figure 1.2. Orthogonal aaRS/tRNA_{CUA} pair and translational machinery. Adapted from *J. Biol. Chem.* **2010**, *285*, 11039-11044.[3]

of 2010.[3] The addition of unnatural amino acids to an organism's genetic code involves three essential components: an orthogonal tRNA-codon pair, an orthogonal aminoacyl-tRNA synthetase (aaRS), and an unnatural amino acid.[5] (See **Fig. 1.2**). The tRNA and aaRS are referred to as orthogonal because they should only recognize each other and not any of the endogenous translational machinery. Thus, an orthogonal tRNA should not bind to an endogenous aaRS and an orthogonal aaRS should not recognize any endogenous tRNAs.

Moreover, the orthogonal tRNA should recognize a degenerate codon that is not commonly used for protein synthesis in the organism of interest. This is because the codon will essentially be changed to encode the unnatural amino acid rather than its traditional function. For example, in the bacterial organism *Escherichia coli,* unnatural amino acid methods have focused on the TAG stop codon (amber stop codon) as it is the least used stop codon with only 7% of all *E. coli* genes ending in TAG.[3] Thus, suppression of this codon through generation of a mutated tRNA with an anticodon CUA should have limited impacts on the *E. coli* organism.[3] Next, an orthogonal aaRS that recognizes the orthogonal tRNA should be evolved via rounds of positive and negative selection steps to recognize the unnatural amino acid of interest and none of the canonical amino acids (See **Fig. 1.3**).[4] In

Figure 1.3. Positive and negative selection cycles for evolution of an orthogonal aaRS.[4]
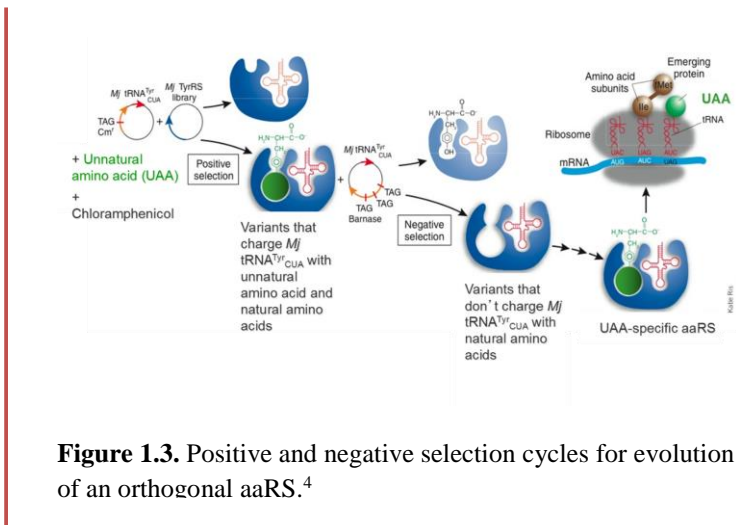
addition, the unnatural amino acid should have a way to enter the cell by either active transport by a membrane-bound protein or simple diffusion. Positive selection is carried out by transforming competent *E. coli* cells with two plasmids. One plasmid carries an aaRS gene from a library and the other plasmid encodes an orthogonal suppressor tRNA that is complimentary to a TAG stop codon and also contains a chloramphenicol resistance gene with a TAG stop codon in the middle of its sequence.[4] The transformed cells are then grown on chloramphenicol plates in the presence of the unnatural amino acid and cells containing aaRS genes which can successfully charge the orthogonal tRNA with an amino

acid and suppress the TAG stop codon will survive.[4] Plasmids from surviving cells are then carried on to the negative selection, which ensures that the aaRS is charging the orthogonal tRNA with the desired unnatural amino acid and not a natural amino acid.[4] This is done by transforming competent *E. coli* cells with one plasmid that contains an aaRS gene that passed positive selection and a second plasmid that contains a barnase gene with its sequence mutated to contain multiple TAG codons and also encodes the same orthogonal tRNA as before.[4] These transformed cells are then grown in culture in the absence of the unnatural amino acid and thus any suppression of TAG codons will be due to the orthogonal aaRS charging the orthogonal tRNA with a natural amino acid.[4] The protein barnase is cytotoxic and thus cells which can suppress TAG stop codons in the absence of the unnatural amino acid will die and be selected against.[4] This cycle of positive and negative selection is often repeated a second time with an additional positive selection step at the end to yield a highly selective aaRS which can charge the orthogonal tRNA with the desired unnatural amino acid.[4] Not performing multiple rounds of selection can result in synthetases which are less selective with regards to the amino acid they charge to the orthogonal tRNA. While less selective, these synthetases can be useful as they can potentially charge multiple similar unnatural amino acids to an orthogonal tRNA which eliminates the need to perform the lengthy selection process for each individual amino acid. The ambryx aminoacyl tRNA synthetase used later in this work did not go through a full selection process and thus will generally recognize derivatives of tyrosine as opposed to one individual unnatural amino acid.

Thus, insertion of an unnatural amino acid into a protein in *E. coli* typically involves the following steps. First, *E. coli* cells are transformed with a plasmid encoding a protein

of interest that has been modified by site-directed mutagenesis to contain a TAG codon at a desired position within the gene and a second plasmid encoding the orthogonal aaRS along with the orthogonal tRNA. Both of these plasmids also contain antibiotic resistance genes to allow for colony selection, in addition to utilizing promoter systems that are independent of one another. This is done so that production of the orthogonal aaRS/tRNA system and the protein of interest can be induced at a controlled time. In this manner, colonies of the transformed cells can be grown up until they reach the log phase of their growth curves. At this time, an unnatural amino acid can be added to the culture along with chemicals that interact with the promoter systems of the plasmids to induce transcription. Once expressed, the mutated orthogonal aaRS should ligate the unnatural amino acid to the orthogonal tRNA. Then, this tRNA can recognize the UAG codon on the mRNA transcript of the protein of interest and add the unnatural amino acid into the growing peptide in a sequence specific fashion. The prime benefit of this methodology is the efficiency of the unnatural protein synthesis that comes from using the cell's normal biosynthetic machinery. Also, if the process is not operating correctly, functional protein should not be produced as the TAG stop codon will not be suppressed, and translation will be terminated at the mutated position. The process is similar for other cell lines with slight modifications to most efficiently make use of a particular organism's biosynthetic machinery.[3] In this manner, modified proteins with unique chemical functionalities specifically incorporated into a desired residue site can be produced efficiently and reliably.

1. C. Protein Immobilization

In addition to the modification of the natural chemical diversity of proteins, intense research has been conducted on developing better methods for incorporating functional

enzymes into the artificial systems found in research labs or industrial settings. As stated previously, enzymes normally do not retain their full catalytic activity in the non-aqueous solvents and high temperatures commonly associated with large scale synthetic or research processes. This inactivation is associated both with protein denaturation caused by elevated temperatures or non-aqueous environments and also the aggregation of proteins which can occur in organic solvents. In addition, free enzymes in solution can be difficult to recover from reaction mixtures and have limited capacity for reuse.[6] Both of these limitations are large drawbacks for industrial processes that rely on high catalytic turnover in the development of economically feasible processes. Protein immobilization seeks to address some of these issues by stabilizing proteins in polymer networks or by attaching proteins to solid supports through a variety of linkages. The immobilization of proteins allows for recyclability, lends stability, and in certain situations can help proteins maintain their functional architecture in reaction conditions that would normally induce denaturation. Furthermore immobilized proteins can lend greater control over reaction times, as a reaction can be stopped simply by filtering a reaction mixture to separate it from the solid support containing catalytic proteins.[6]

Common immobilization strategies include entrapment, encapsulation, support based immobilization, and self-immobilization.[7] Entrapment involves a trapping of enzymes in a porous solid support such as a network of polymers.[6] Entrapment allows the trapped enzyme to carry out its catalytic functions while avoiding contact with any external hydrophobic species such as gas bubbles or organic solvents.[8] The main drawback for this strategy is that proteins can leak out of the porous solid support and there usually are low levels of protein loading.[7] Encapsulation is a method that also attempts to help preserve

enzyme stability through protecting enzymes from their external environment. Rather than using porous networks however, encapsulation traps enzymes in micelles. Unfortunately this method suffers from limitations of what masses can be transferred into the micelle and thus has limited use for the catalysis of large substrates.[7] Support based immobilization is probably the most diverse and widely used category. This method involves attaching a protein to a derivatized solid support through either an ionic or covalent bond. This bonding can increase rigidity and increase a protein's stability in a reaction mixture. One of the most common strategies involves forming covalent bonds with lysine residues as it is a relatively common amino acid, is fairly reactive, and is typically surface exposed in proteins.[7] Thus a variety of chemical strategies have been devised to form covalent bonds between the amino group of lysine and chemically derivatized solids. This functional approach has also been

Amino acid	Reactive residue	Method of attachment	Reaction
ε-amino group of lysine and N-terminal amino group	—NH$_2$	Diazotization	—N=N—Protein
		Peptide bond formation	—C(=O)—NH·Protein
		Arylation	(aryl ring) NH·Protein, —NO$_2$, O$_2$N
		Alkylation	—CH$_2$—NH—Protein
		Schiff-base formation	—CH=N—Protein
		Amidination	NH$_2^+$ / —C—NH·Protein
Sulfhydryl of cysteine	—SH	Alkylation	—CH$_2$—S—Protein
		Thio-disulfide interchange	—S—S—Protein
		Mercury enzyme interaction	—Hg—S—Protein
Carboxyl group of aspartate and glutamate and C-terminus carboxyl group	—COOH	Peptide bond formation	—C(=O)—NH·Protein
Phenolic of tyrosine	(ring)—OH	Diazotization	—N=N—(ring with Protein, HO)
Imidazole of histidine	HN—(imidazole)=N	Diazotization	—N=N—(imidazole with Protein, HN)=N

Table 1.1. Residues targeted in various covalent immobilization strategies. Adapted from *Microchimica Acta*. **1998**, *128*, 127-143.[9]

copied with a variety of other amino acid residues (see **Table 1.1**).[9] The prime benefit of this type of immobilization is the generation of a stable and reliable protein linkage that can increase protein rigidity and allow for reuse. This method does present a variety of complications however; as the covalent bonds formed are not specific with regards to

which residues react. Also, proteins usually end up widely dispersed on the solid support which can reduce their specific activity by a factor of 10 or more.[7] The last major method of immobilization, self-immobilization, tries to address this issue by using similar reactions or cross-linking reagents to link proteins to each other. The main disadvantages of this technique are that it only applies to crystallisable proteins and requires a great deal of purification.[7]

As can be seen above, protein immobilization strategies can present their own unique challenges. The most common reaction schemes involve covalent linkage with a certain amino acid, so there is a lack of specificity in many strategies due to the large number of residues that could react in any given protein (See **Fig 1.4**). Indeed, immobilization itself can sometimes disrupt protein function due to attachment of proteins on the
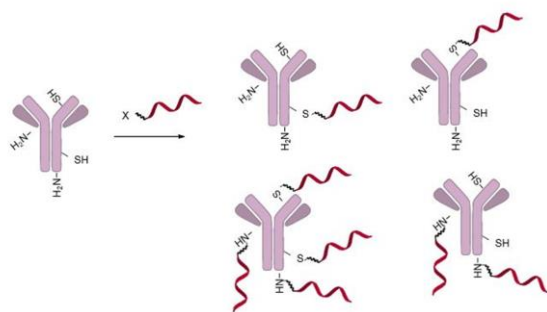
Figure 1.4. General problems associated with protein conjugation. The strategy above is not selective with regards to where the functional group attaches to the antibody protein nor with regards to how many functional groups are conjugated to one protein.

immobilization solid support through several amino acid residues. A commonly related problem is the loss of protein activity in certain immobilization reactions due to attachments that interfere with the active site.[9] Thus, a strategy which could provide a site-specific protein linkage far away from a protein's catalytic core could drastically increase the efficiency of protein immobilization. This problem is an excellent example of where two emerging techniques in chemical biology could be combined. As discussed earlier, unnatural amino acids have unique functionalities and can be inserted into a specific

position far away from a protein's catalytic core.[10] Thus, a reaction scheme involving covalent linkage to an inserted unnatural amino acid holds the potential for site-specific and efficient protein immobilization as desired above with greatly reduced effects on protein activity. Interesting potential candidates for this type of immobilization include unnatural amino acids that include either an azide or terminal alkyne in their modified side chains. As stated earlier, neither of these of these functional groups exist in the canonical twenty amino acids and thus an immobilization protocol that involved a selective reaction with one of these groups would generate a single site-specific protein linkage as desired above. Furthermore, the Huisgen 1, 3 dipolar cycloaddition between an azide group and a terminal alkyne has been well documented and is a hallmark of the emerging field of "click chemistry" which emphasizes quick, reliable and high yielding reactions that mimic nature by joining together small molecular units.[11] (See **Fig. 1.5**) For example, a solid support derivatized with a long chain hydrocarbon group that contained a terminal alkyne could form a
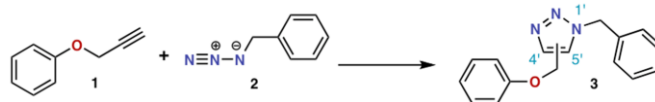
Figure 1.5. Example Huisgen 1, 3 dipolar cycloaddition.

covalent bond through this cycloaddition with an azide group on an unnatural amino acid that had been synthesized and inserted into a protein of interest. Indeed, in a recent study, the use of click chemistry was investigated for the immobilization of soluble proteins onto a derivatized glass slide through an unnatural amino acid generated by post-translational modification.[12] Researchers were able to post-translationally alkylate cysteine residues of various proteins such as GFP with farnesyl pyrophosphate derivatives that contained terminal alkyne or azide groups.[12] These proteins were then immobilized on derivatized glass slides either through a "click chemistry" reaction or a Staudinger ligation.[12] As a

specific example, GFP that had been alkylated with a farnesyl derivative containing a terminal alkyne was immobilized through the Huisgen 1,3 dipolar cycloaddition onto a glass slide that had been derivatized with PEG-azide groups.[12] The success of the immobilization reaction was confirmed by the measurement of fluorescent activity from the GFP on the slide after it had been washed repeatedly.[12] In addition, this fluorescent activity was found to only decrease slightly over two days of measurements.[12] This indicated that immobilized GFP could retain its activity and stability throughout the immobilization process and subsequent storage in buffer.[12] Thus, the combination of these two emerging techniques presents exciting opportunities for new selective bioconjugations that can vastly expand the utility of enzymes in artificial systems. Along this line of thinking, systems have been developed that utilize the site-specific incorporation of unnatural amino acids for protein immobilization onto M-20 Dynabeads but are less efficient because they utilize cell-free protein synthesis.[13] Thus, adapting amber stop codon suppression technology to insert reactive unnatural amino acids for immobilization strategies rather than post-translationally modifying desired residues has the potential to drastically improve the efficiency of this process. Indeed, this combination of techniques has been used to immobilize calmodulin with an unnatural amino acid at a defined residue onto carbon nanotubes and nanoparticles via the Staudinger-Bertozzi ligation.[14,15] In addition, this combination of techniques has been used to indirectly immobilize proteins on streptavidin derivatized gold surfaces via a click reaction between a site-specifically incorporated unnatural amino acid and biotin which then reacts with the streptavidin derivatized surface.[16] Importantly, however, no study has analyzed whether these unnatural amino acid immobilization strategies can be improved through the targeting of specific

regions in proteins nor developed a generalized immobilization protocol which would allow for this technique to be applied to a wide range of proteins. Finally, a more complete understanding of the optimal immobilization conditions required when using unnatural amino acid technology for protein immobilization is crucial to fully developing this powerful system.

1. D. Microwave-Assisted Reactions and Biocatalysis

Another emerging technique that holds potential for increasing the effectiveness of enzymes outside of natural systems is the field of microwave chemistry. Microwave-assisted organic synthesis originally grew out of a desire to find better methods of heating than conventional heating modes. Much maligned, conventional heating modes depend on transferring heat to the walls of a reaction vessel before the heat is then transferred to the reaction solvent.[17] These wall effects lead to heating modes that are slow and largely inefficient due to uneven heating. In contrast, heating by microwave radiation allows for much more efficient heating as microwave irradiation induces the molecular motion of ions or dipoles in solution when they align with the oscillating electromagnetic field.[18] (See **Fig. 1.6**) By avoiding wall effects, microwave heating allows for faster and

Figure 1.6. Temperature profiles of reaction vessels heated by microwave irradiation (left) and by oil bath (right). Adapted from *Angew. Chem. Int. Ed.* **2004**, *43*, 6250-6284.[17]

more even modes of heating and has been shown to increase yields and decrease wait times for various organic reactions when compared to controls heated in a conventional manner.[18]
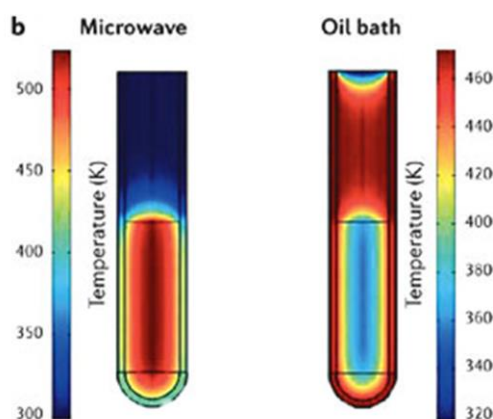
More recently, microwave irradiation has been investigated for potential enhancements in enzyme catalysis, especially with regards to hyperthermophilic enzymes (those coming from organisms which typically grow at and above 90 °C).. These enzymes are isolated from various hyperthermophile organisms that live in thermal vents or various other harsh natural environments. Thus, these enzymes are typically not active below 40 °C and don't begin to denature until temperatures much higher than what would denature mesophilic enzymes (those coming from organisms which typically grow between 20 and 45 °C).[19] In a recent study, it was shown that various hyperthermophilic enzymes exhibited up to four orders of magnitude increases in catalytic activity under microwave irradiation when compared to thermal controls.[19] This effect is thought to be potentially a result of the highly dipolar nature of enzymes and the increases in conformational flexibility that can come with dipole alignment in the oscillating electromagnetic field.[19] This is an extremely interesting development for adapting enzymes to artificial systems as not only are these hyperthermophilic enzymes able to maintain their stability under microwave conditions but can even display increased catalytic activity.

Due to the nature of the technique, microwave assisted biocatalysis also lends itself to combination with the other emerging immobilization methods presented in this paper. Unnatural amino acids could be inserted into hyperthermophilic enzymes in the same manner as discussed earlier to provide functional handles for directed and site-specific bioconjugations. Then as stated previously, microwave irradiation has displayed the potential to dramatically improve the efficiency of various organic reactions such as the "click chemistry" cycloaddition proposed earlier for protein immobilization through an unnatural amino acid. Performing this reaction scheme under microwave irradiation could

drastically increase the efficiency of the immobilization process while decreasing reaction time. Furthermore, successfully immobilized enzymes that retain or even display enhancements in their catalytic activity under microwave heating could then be used to selectively catalyze reactions using this heating mode. Thus, an integrative approach could combine the increases in stability associated with immobilization through an unnatural amino acid with the increases in efficiency and catalytic activity associated with microwave-assisted reactions.

1. E. Conclusions

Thus, emerging methods that allow for the modification of enzymes to facilitate their increased integration into artificial systems have the potential to expand the boundaries of what has been traditionally possible in research labs and industrial processes. Unnatural amino acids can add functionalities and chemical properties to proteins that are not found in nature. Furthermore, protein immobilization can increase protein stability and activity in reaction conditions such organic solvents and high temperatures that normally induce denaturation or decreases in catalytic efficiency. Lastly, microwave irradiation can increase not only the efficiency of organic reactions, but also the catalytic activity of hyperthermophilic enzymes. Ultimately, novel methods that can effectively combine the incredible efficiency and utility of natural systems with the conventional techniques used throughout industry and chemical research hold the potential to lead scientists and engineers towards more efficient methods of chemical synthesis.

Chapter 1 References

1. Berg, J.; Tymoczko, J.; Stryer, L. *Biochemistry*; Macmillan: New York, **2010**.
2. Lowe, D. Natural Born Chemists. *Chemistry World*. [Online] **2013**, http://www.rsc.org/chemistryworld/2013/10/natural-born-chemists-derek-lowe-pipeline (accessed Jan 13, 2014).
3. Schultz, P. G.; Young, T. S. Beyond the Canonical 20 Amino Acids: Expanding the Genetic Lexicon. *J. Biol. Chem*. **2010**, *285*, 11039-11044.
4. Wang, L.; Xie, J.; Schultz, P. G. Expanding the Genetic Code. *Annu. Rev. Biophys. Biomol. Struct*. **2006**, *35*, 225–249.
5. Wang, W.; Takimoto, J.; Louie, G.; Baiga, T.; Noel, J.; Lee, K.; Slesinger, P.; Wang, L. Genetically encoding unnatural amino acids for cellular and neuronal studies. *Nature Neuroscience*. **2007**, *10*, 1063-1072.
6. Mateo, C.; Palomo, J.; Fernandez-Lorente, G.; Guisan, J.; Fernandez-Lafuente, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology*. **2007**, *40*, 1451–1463.
7. Brady, D.; Jordaan, J. Advances in enzyme immobilization. *Biotechnol. Lett*. **2009**, *31*, 1639–1650.
8. Elgren, T.; Zadvorny, O.; Brecht, E.; Douglas, T.; Zorin, N.; Maroney, M.; Peters, J. Immobilization of Active Hydrogenases by Encapsulation in Polymeric Porous Gels. *Nano Lett*. **2005**, *5*, 2085–2087.
9. Rao, S.; Anderson, K.; Bachas, L. Oriented immobilization of proteins. *Microchimica Acta*. **1998**, *128*, 127-143.
10. Noren, C.; Anthony-Cahill, S.; Griffith, M.; Schultz, P. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science*. **1989**, *244*, 182-188.
11. Huisgen, R. Kinetics and Mechanism of 1, 3-Dipolar Cycloadditions. *Angew. Chem. Int. Ed*. **1963**, *2*, 565-598.
12. Gauchet, C.; Labadie, G.; Poulter, C. Regio- and Chemoselective Covalent Immobilization of Proteins through Unnatural Amino Acids. *J. Am. Chem. Soc*. **2006**, *128*, 9274-9275
13. Smith, M.; Wu, J.; Varner, C.; Bundy, B. Enhanced protein stability through minimally invasive, direct, covalent, and site-specific immobilization. *Biotechnol. Prog*. **2013**, *29*, 247−254.
14. Yoshimura, S.; Khan, S.; Ohno, S.; Yokogawa, T.; Nishikawa, K.; Hosoya, T.; Maruyama, H.; Nakayama, Y.; Takeyasu, K. Site-specific attachment of a protein to a carbon nanotube end without loss of protein function. *Bioconjugate Chem*. **2012**, *23*, 1488−1493.
15. Ikeda-Boku, A.; Kondo, K.; Ohno, S.; Yoshida, E.; Yokogawa, T.; Hayashi, N.; Nishikawa, K. Protein fishing using magnetic nanobeads containing calmodulin site-specifically immobilized via an azido group. *J. Biochem*. **2013**, *154*, 159−165.
16. Seo, M.; Han, J.; Jin, Z.; Lee, D.; Park, H.; Kim, H. Controlled and oriented immobilization of protein by sitespecific incorporation of unnatural amino acid. *Anal. Chem*. **2011**, *83*, 2841−2845.

17. Kappe, C. Controlled Microwave Heating in Modern Organic Synthesis. *Angew. Chem. Int. Ed.* **2004**, *43*, 6250-6284.

18. Herrero, M.; Kremsner, J.; Kappe, C. Nonthermal Microwave Effects Revisited: On the Importance of Internal Temperature Monitoring and Agitation in Microwave Chemistry. *J. Org. Chem*. **2008**, *73*, 36-47.

19. Young, D.; Nichols, J.; Kelly, R.; Deiters, A. Microwave Activation of Enzymatic Catalysis. *J. Am. Chem. Soc*. **2008**, *130*, 10048–10049.

Chapter 2: Development of unnatural amino acid immobilization methodology using a GFP model system

2. A. Insertion of unnatural amino acids into GFP

Green Fluorescent Protein (GFP) was selected as a model protein for studying protein immobilization through an unnatural amino acid for two primary reasons. Its fluorescent properties make it an ideal reporter protein as a solid support can be quickly analyzed under ultra-violet (UV) irradiation to determine whether GFP is still attached to the solid after the immobilization reaction and washes to remove non-covalently bonded protein. In addition, plasmids encoding GFP with a TAG stop codon mutated into various positions were readily available, having been developed for other experiments. As examined in the introduction, this TAG codon is necessary for inserting unnatural amino acids when using an orthogonal synthetase/tRNA system. For this study, plasmids with the TAG codon at residues 3, 133, and 151 in the functional protein were selected (See **Fig. 2.1**). Selecting
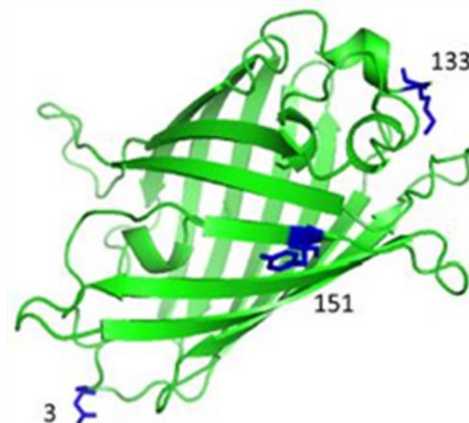
Figure 2.1. GFP positions targeted in this study. Adapted from PDB: 1EMA.[1]

these residues allowed for the study of potential variations in immobilization efficiency depending on the region where the functional handle (i.e. the unnatural amino acid) for immobilization was inserted. Residue 3 is located at the N-terminus of GFP and as such is in a loosely structured region with a high degree of conformational freedom. Residue 151

is located on the beta-barrel of GFP and thus is in a highly structured region with little conformational freedom. Lastly, residue 133 is located on a turn in the protein and therefore displays intermediate conformational flexibility between residue 3 and 151. Once these residues had been selected, the unnatural amino acid, *p*AzF, was selected for incorporation into the GFP mutants. *p*AzF is shorthand for *para*-Azidophenylalanine meaning that it is a derivative of phenylalanine (F in the one letter amino acid code) and has an azide moiety in the *para* position on the aromatic ring (See **Fig. 2.2**). This unnatural amino acid was chosen so that mutant proteins could participate in a bioorthogonal 1, 3-dipolar Huisgen cycloaddition with the selected solid support, which would be derivatized with alkyne moieties. Bioorthogonal reactions do not interfere with native biochemical processes and do not display cross reactivity with functional groups found in natural systems.
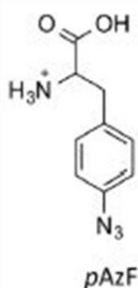
Figure 2.2. *p*-Azidophenylalanine unnatural amino acid (*p*AzF). Adapted from *Bioconjugate Chem.* 2014, *25* (11), 1916-1920.[2]

Furthermore, the unnatural synthetase *p*CNF-aaRS was selected as it was evolved to specifically incorporate the *p*AzF unnatural amino acid. Importantly, if this methodology was successful, other unnatural amino acid and solid support combinations could be used such as an unnatural amino acid with an alkyne functionality paired with a solid support derivatized with azide groups. In addition, the Young Lab has also been investigating the use of Glaser-Hay couplings between two alkyne moieties as a bioorthogonal reaction. Thus, this methodology could potentially even be used to immobilize proteins containing unnatural amino acids with alkyne functionalities on solid supports derivatized with alkyne moieties.

Thus the unnatural amino acid, *p*AzF, was inserted in the specified positions on the GFP 3, 133, and 151 mutants using the method described in the introduction and the *p*CNF synthetase protein, evolved to charge orthogonal tRNAs recognizing the TAG stop codon with our unnatural amino acid. (See Experimental Section) SDS-PAGE analysis was then used to assess successful incorporation of the unnatural amino acid. For each mutant, minimal functional protein was observed in the absence of the unnatural amino acid, indicating that the

Figure 2.3. SDS-PAGE analysis of GFP-151-*p*AzF expression. No protein is present in the absence of the UAA (lane 1) suggesting successful incorporation of the UAA (lane 2). Adapted from *Bioconjugate Chem.* **2014**, *25* (11), 1916-1920.[2]

inserted stop codon was successfully read through and the unnatural amino acid was incorporated in the given position when it was present in the expression (See GFP-151-*p*AzF in **Fig. 2.3**).

2. B. Optimization of solid support and immobilization conditions

Initially, a tritylchloride modified polystyrene resin, an epoxide derivatized Sepharose 6B resin, and a carboxy-modified Tenta-Gel resin were investigated to determine the best material for these immobilization reactions. Tritylchloride modified polystyrene resin is highly hydrophobic, carboxy-modified Tenta-Gel resin is moderately hydrophobic, and epoxide derivatized Sepharose 6B resin is moderately hydrophilic. All of these resins contained functional groups that could easily react with molecules

containing amines or alcohols to facilitate derivatization (Trityl group-polystyrene resin, epoxide group-Sepharose 6B resin, carboxylic acid group-Tenta-Gel resin). Exploiting this reactivity, each solid support material was derivatized with alkynyl linkers of various lengths that could react with the azide-containing unnatural amino acid on the GFP surface (See Experimental Section). In addition, to investigate the effect of changing the length of the carbon tether between the alkyne group and the solid support surface, each resin was reacted with propargyl alcohol, 1-hexynol, or 1-undecynol (See **Fig. 2.4**). Loadings of the various linkers onto the resins were comparable between the different solid supports and alkynyl linkers, and were tested by cleaving 15 mg samples of each resin/linker combination
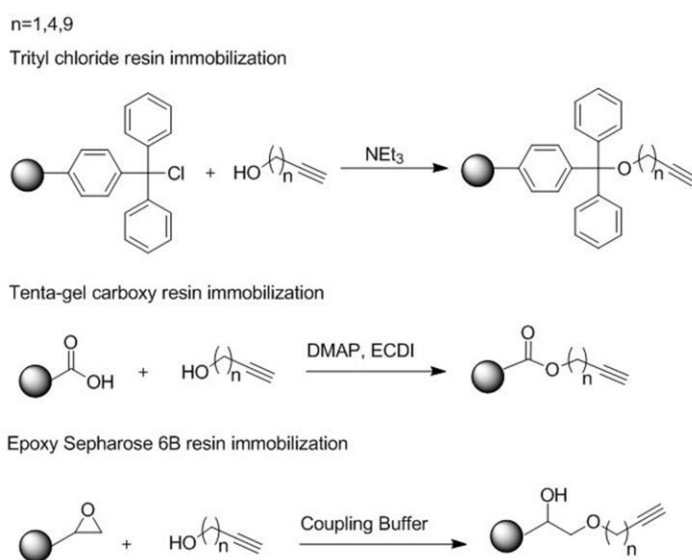
Figure 2.4. Immobilization reaction schemes for derivatizing Trityl chloride resin, Tenta-gel carboxy resin, and Epoxy Sepharose 6B resin with variable length alkynyl linkers.

and investigating the products by TLC and GC/MS analysis. Gratifyingly, each resin sample displayed an approximate loading of 0.6-0.9 mmol/g of immobilized alkyne. This relatively low and consistent loading observed was beneficial for two reasons. First, it facilitated comparisons of immobilization efficiency between different types of resin. Second, low resin loading is a necessary element for preserving the activity of an immobilized protein as solid supports with high linker loadings can force immobilized

proteins to come together in close proximity and begin to aggregate, destroying protein function.

After all the alkynyl linkers had been immobilized on the respective solid supports, each resin was subjected to click conditions with GFP-151-*p*AzF protein and a copper catalyst system for 16 h at 4 °C in an attempt to immobilize the protein on each solid support through the unnatural amino acid (See Experimental Section and **Fig. 2.5**). Protein concentrations
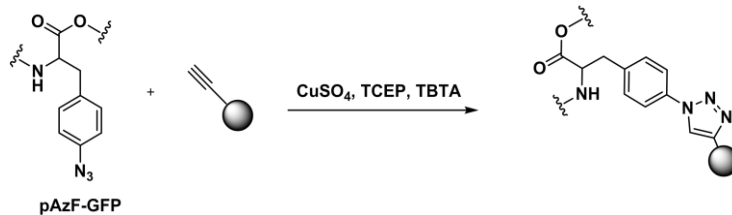
Figure 2.5. Typical immobilization reaction scheme between a *p*AzF mutant and resin derivatized with an alkynyl linker.

were measured beforehand and taken into account with all the previously measured resin loadings to ensure that each reaction had approximately 12 μmol of alkyne and 35 μg of protein. In addition, the polystyrene resin and Tenta-gel resin are more hydrophobic than the Sepharose resin, so 10% DMSO was added to reaction mixtures including either the polystyrene resin or the Tenta-gel resin in order to prevent excessive aggregation of the solid support. After 16 hours, the resin samples were transferred to spin columns and washed 5 times with PBS buffer before being assessed on a plate reader for the presence of immobilized GFP. The fluorescence intensity from immobilized GFP was consistently higher on the Sepharose resin, while little to no fluorescence was consistently observed on the Tenta-gel or polystyrene resins (See **Fig. 2.6**). This decreased immobilization efficiency observed on the Tenta-gel and polystyrene resins is most likely due to the aforementioned high hydrophobicity of these two solid supports and their subsequential aggregation in an aqueous solvent, blocking access of the reactive surface to the protein. It

is possible that in the future the efficiency of immobilization onto the polystyrene and Tenta-Gel resins could be improved by optimizing organic solvent ratios that could prevent resin aggregation but not denature the protein. However, given the successful Sepharose immobilizations, these results clearly indicated that immobilized GFP could retain its functional structure throughout our immobilization process. Furthermore, control

Figure 2.6. Fluorescence data form propargyl alcohol derivatized-sepharose 6B resin incubated with wt-GFP (left) and GFP-151-*p*AzF (right). Adapted from *Bioconjugate Chem.* **2014**, *25* (11), 1916-1920.[2]

reactions were performed where wild-type GFP with no azide moiety was incubated with derivatized resin under identical reaction conditions to the reactions analyzed above and where GFP-151-*p*AzF was incubated with derivatized resin in the absence of copper catalyst (the Huisgen 1, 3-dipolar cycloaddition is exceedingly slow in the absence of catalyst). Little to no fluorescence was observed on either control resin sample after washing with PBS buffer, confirming that the manner of immobilization was the covalent attachment through the "click" reaction between *p*AzF and the alkynyl linker rather than non-covalent hydrophobic interactions between GFP molecules and hydrophobic regions of the solid support (See **Fig. 2.6**).

2. C. Effect of alkynyl linker length on immobilization efficiency

The effect of linker length on immobilization efficiency was then analyzed by comparing amounts of immobilized fluorescence when GFP-151-*p*AzF was incubated with sepharose 6B resin samples with variable length alkynyl linkers. A trend was observed where increased linker length was correlated with decreased immobilization efficiency and this effect persisted even when reaction times were extended (See **Fig. 2.7**). Two factors likely contribute to this observed trend. Linkers of longer length (increased methylene units) are

Figure 2.7. Fluorescence data of GFP-151-*p*AzF immobilized with sepharose 6B resin samples derivatized with variable length alkynyl linkers. The maximum level of fluorescence was observed with propargyl alcohol derivatized sepharose resin and minimal fluorescence was observed for control experiments with WT-GFP. Error bars represent standard deviations from 3 separate experiments. Adapted from *Bioconjugate Chem.* **2014**, *25* (11), 1916-1920.[2]

inherently more hydrophobic and thus could tend to aggregate in aqueous solutions inhibiting their proper interaction with the azide moiety of GFP-151-*p*AzF. Furthermore, longer linkers have increased conformational freedom and it is possible that this can inhibit proper orientation of the alkyne moiety for conjugation to the protein.

2. D. Effect of UAA context on immobilization efficiency

Once optimal immobilization conditions had been developed, sepharose 6B resin derivatized with propargyl alcohol was then incubated with GFP-3-*p*AzF, GFP-133-*p*AzF, and GFP-151-*p*AzF to determine whether the position of the unnatural amino acid within the protein had an effect on immobilization efficiency. These mutants were expressed using the procedures as described above and utilizing plasmids with TAG mutations at positions 3, 133, 151 respectively (See Experimental Section). In addition, each protein was diluted to identical concentrations (See Experimental Section). As examined previously, these residues are all surface exposed to facilitate the conjugation reaction but each is located in a different position in the protein with variable rotational flexibility. To review, residue 3,

Figure 2.8. Fluorescent emission spectra of GFP mutants compared to wild-type GFP. Adapted from *Bioconjugate Chem.* **2014**, *25* (11), 1916-1920.[2]
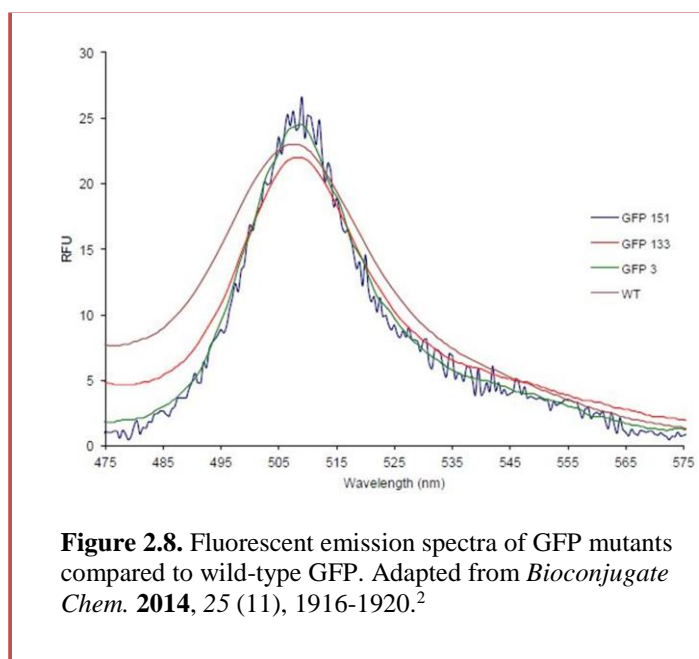
which is located in the largely unstructured N-terminal region, has the greatest conformational freedom while residue 151, which is located on the β-barrel of GFP, has the least conformational freedom. Residue 133, which is located on a loop region of the β-barrel, falls in between with intermediate conformational freedom (See **Fig. 2.1**). In addition, fluorescent spectra of all the mutants were taken to ensure that fluorescence readings could be used to reliably compare immobilization efficiencies between the different mutants and that observed

differences were not due to differences between the mutants' emission spectra (See **Fig. 2.8**). Each GFP mutant was then subjected to the same click immobilization conditions and worked up as described above (See Experimental Section). Fluorescence readings on a plate reader were then taken from each washed sample. A difference in fluorescence intensities was consistently observed between the different mutants, implying variable immobilization efficiencies that are dependent on unnatural amino acid context. GFP-151-*p*AzF reproducibly displayed the highest degree of immobilization followed by GFP-133-*p*AzF, and GFP-3-*p*AzF. Minimal fluorescence was observed in a control reaction where the copper catalyst was removed (See **Fig. 2.9**). As examined
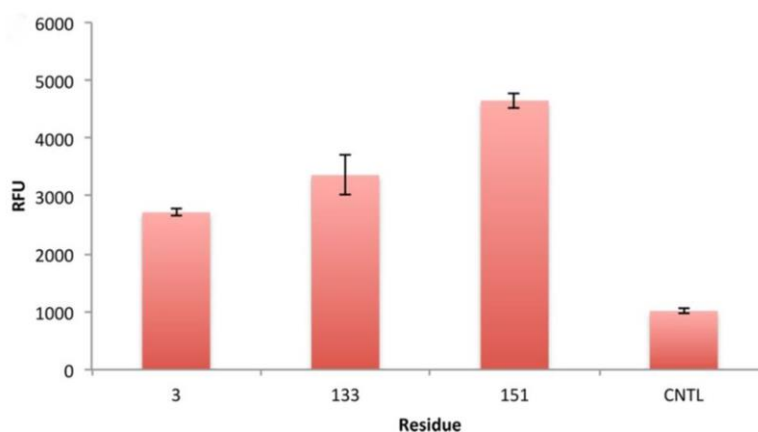
Figure 2.9. Fluorescence data of GFP-3-*p*AzF, GFP-133-*p*AzF, and GFP-151-*p*AzF mutants immobilized onto propargyl alcohol derivatized sepharose 6B resin. All error bars represent standard deviations from three independent measurements. Adapted from *Bioconjugate Chem.* **2014**, *25* (11), 1916-1920.[2]

in detail above, these residues were selected for investigation based on their positions in different protein environments. Thus, the trend observed for immobilization efficiency with regards to unnatural amino acid context matches the trend observed for immobilization efficiency with regards to linker length where moieties with the least conformational freedom correlated to the largest immobilization efficiency. Clearly, when bringing two large polymers together for conjugation (solid supports and polypeptide chains), these results indicate that minimal conformational freedom on both sides of the

reaction is ideal. Indeed, increased rigidity probably serves to prevent the aggregation or interaction of reactive groups with the polymer they are located on rather than the other polymer in the conjugation reaction. Thus, when using an unnatural amino acid as a bioorthogonal handle for protein immobilization, this work indicates that the conformational freedom of the mutated site must be considered in addition to surface accessibility and non-interference with protein function.

2. E. Retention of fluorescence in organic solvents

Finally, to investigate whether GFP immobilized using this methodology was truly more stable and resistant to denaturation than free GFP, retention of fluorescence in various organic solvents was measured for both free GFP and immobilized GFP. For these studies, GFP-151-*p*AzF was used both in free form and immobilized to propargyl alcohol derivatized sepharose 6B resin. As DMF is fairly miscible with water, both free and immobilized resin incubated for 2 hours in solutions with varied percentages of DMF (25%-100%) and afterwards fluorescence was measured on a plate reader as in the preceding experiments. Free GFP

Figure 2.10: A.) Fluorescence data of resin-bound GFP-151-*p*AzF and free GFP-151-*p*AzF in varying percentages of DMF. B.) Fluorescence data of resin-bound GFP-151-*p*AzF and free GFP-151-*p*AzF in 100% solutions of various organic solvents. All error bars represent standard deviations from three independent measurements. Adapted from *Bioconjugate Chem.* **2014**, *25* (11), 1916-1920.[2]

fluorescence was observed to rapidly decline in solutions of 50% DMF and above which was in agreement with literature precedent (See **Fig. 2.10**).[3] Expanding on this initial study, free GFP-151-*p*AzF and immobilized GFP-151-*p*AzF were then both incubated for 2h in 100% solutions of hexanes, ethyl acetate, and dichloromethane and afterward fluorescent intensities were measured on a plate reader. In agreement with the DMF study, immobilized GFP was able to retain significant activity in organic solutions whereas free

GFP displayed very little activity and as expected was largely denatured in these organic solutions (See **Fig. 2.10, Fig. 2.11**). Lastly, to investigate whether these increases could have been caused by

Figure 2.11: Representative images of resin-bound GFP-151-*p*AzF and free GFP-151-*p*AzF in 100% solutions of various organic solvents under UV irradiation. Top.) Resin-bound GFP-151-*p*AzF. Bottom.) Free GFP-151-*p*AzF. Adapted from *Bioconjugate Chem.* **2014**, *25* (11), 1916-1920.[2]
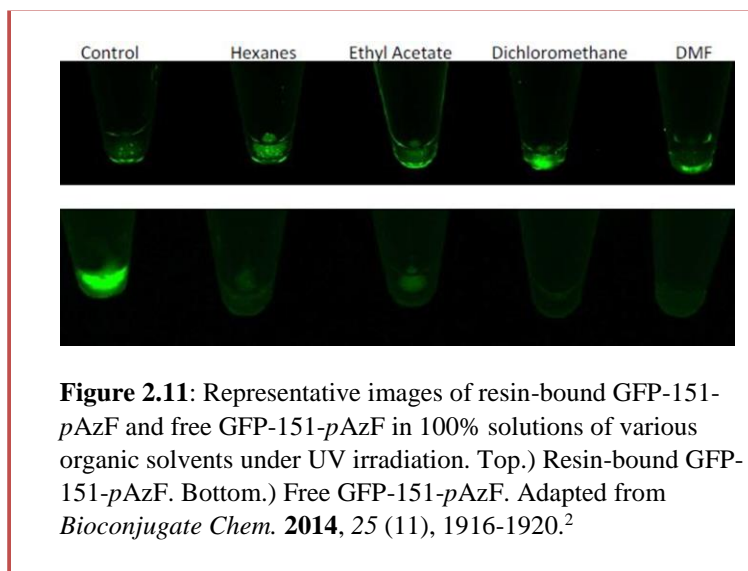
a solvation sphere that remained around the protein due to the solid support, protein samples were incubated for 48 hours and subjected to sonication. In both, cases immobilized GFP retained its fluorescence while free GFP remained largely denatured. Importantly, this increase in stability is not unique to this immobilization methodology, and is one the desired features of any protein immobilization technique. However, these organic solvent investigations do indicate that this immobilization methodology can produce similar increases in protein stability as other methods with the added benefit of being site-selective. Thus, this methodology could be particularly useful for proteins that are sensitive to the orientation of immobilization.

2. F. Conclusions

Ultimately, this work reflects the development of a new methodology of protein immobilization that can conjugate proteins to a solid support in a site-selective and stable manner in order to maintain functionality in conditions that would normally induce denaturation if the proteins were in solution. The unnatural amino acid *p*AzF was chosen due to its ability to participate in a bioorthogonal reaction with a terminal alkyne in the presence of copper catalyst and general inertness to other reactive groups. For the solid support, it was found that a resin with minimal hydrophobicity such as the sepharose 6B resin was crucial to prevent aggregation in the aqueous solution used for the immobilization reaction. For selection of alkynyl linker, aggregation was also a concern and it was found that short linking groups with reduced conformational freedom produced the highest immobilization efficiencies. Then, when investigating the effect of UAA context on immobilization efficiency, an identical trend was found where residues with reduced conformational freedom produced the highest immobilization efficiencies. Thus, when considering an UAA context for immobilization, one must consider not only proximity to the active site but also the relative conformational freedom of the mutated residue. Lastly, GFP immobilized in this manner was found to retain its fluorescence even in conditions that completely denatured solution phase GFP. Thus, this model system represents a new technique for immobilization and also offers guidelines for applying the methodology to any desired protein or enzyme. In this manner, desired enzymes could be examined structurally to determine optimal sites for unnatural amino acid insertion and then site-selectively immobilized using the procedures outlined above for use in environments normally unsuitable for proteins. Therefore, in conclusion, protein immobilization through

an unnatural amino acid could drastically increase the use of enzymes in industrial catalysis and other harsh reaction conditions and as a result increase the efficiency of numerous synthetic processes relevant to medicine, engineering and energy.

Chapter 2 References

1.) Ormö, M.; Cubitt, A. B.; Kallio, K.; Gross, L. A.; Tsien, R. Y.; Remington, S. J. Crystal structure of the Aequorea Victoria green fluorescent protein. *Science*. **1996**, *273*, 1392−1395.
2.) Raliski, B.K.; Howard, C.A.; Young, D.D. Site-Specific Protein Immobilization Using Unnatural Amino Acids. *Bioconjugate Chem.* **2014**, *25* (11), 1916-1920.
3.) Raghunathan, G.; Sokalingam, S.; Soundrarajan, N.; Munussami, G.; Madan, B.; and Lee, S. A comparative study on the stability and structure of two different green fluorescent proteins in organic cosolvent systems. *Biotechnol*. *Bioprocess Eng*. **2013**, *18*, 342−349.

Experimental Section

General expression of GFP with pAzF unnatural amino acid

A pET-GFP-TAG variant plasmid (0.5 µL) was co-transformed with a pEVOL-*p*CNF evolved synthetase plasmid (0.5 µL) into Escherichia coli BL21 (DE3) cells using an Eppendorf eporator electorporator. The cells were then plated and grown on LB agar in the presence of chloramphenicol (34 mg/mL) and ampicillin (50 mg/mL) at 37˚C overnight. One colony was then used to inoculate LB media (4 mL) containing both ampicillin and chloramphenicol. The culture was incubated at 37˚C overnight and used to inoculate an expression culture (10 mL LB media, 50 mg/mL Amp, 34 mg/mL Chlor) at an OD600 0.1. The cultures were incubated at 37˚C to an OD600 between 0.6 and 0.8 at 600 nm, and protein expression was induced by addition of *p*AzF (100 µL, 100 mM) and 20 % arabinose (10 µL) and 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; 10 µL). The cultures were allowed to shake at 30˚C for 16-20 h then centrifuged at 5,000 rpm for 10 minutes and stored at -80˚C for 3 hours. The cell pellet was re-suspended using 500 µL of Bugbuster (Novagen) containing lysozyme, and incubated at 37˚C for 20 minutes. The solution was transferred to an Eppendorf tube and centrifuged at 15,000 rpm for 10 minutes, then the supernatant was poured into an equilibrated His-pur Ni-NTA spin (Qiagen) column with of nickel resin (200 µL) and GFP was purified according to manufacturer's protocol. Purified GFP was analyzed by SDS-PAGE (BioRad 10% precast gels, 150V, 1.5h), and employed without further purification. Protein concentrations were determined both by a BCA assay and fluorescence measurements.

Immobilization of Alkynes onto Trityl Chloride Resin

To flame dried vial, trityl chloride resin (200 mg, 0.36 mmol, 1 equiv.) and dichloromethane (5 mL) were added. The resin was swelled at room temperature with gentle stirring for 15 min. Alkyn-ol (1.2 equiv.) was added to reaction, followed by triethylamine (0.2 equiv). The mixture was stirred at room temperature for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each). The resin was swelled in DCM and dried under vacuum for 45 min before further use. Resin (15 mg) was added to an Eppendorf tube followed by trifluoroacetic acid (2%, 200 μL). The mixture was shaken at room temperature for 1 h, then subjected to thin layer chromatography, GC/MS, and mass difference analysis to confirm resin loading.

Immobilization of Alkynes onto Tenta-Gel Carboxy Resin

To a vial, Tenta-gel carboxy resin (200 mg, 0.046 mmol, 1 equiv.) and dichloromethane (3 mL) were added. The resin was swelled at 30˚C for 15 minutes. Alkyn-ol (5 equiv.) was added to the swelled resin, followed by 4-dimethylaminopyridine (DMAP, 0.2 equiv.) and 1-Ethyl-3-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (ECDI, 5 equiv.). The reaction was stirred at room temperature for 16 h. The resin was transferred to a syringe filter and washed with dichloromethane and methanol (5 alternating washes with 3 mL each). The resin was swelled in DCM and dried under a vacuum for 45 minutes before further use. A tetrahydrofuran/methanol solution (4:1, 200 μL) was added to an Eppendorf tube along with potassium carbonate (25 mg) followed by resin (15 mg). The mixture was shaken for 16 h at 37˚C. The product was then subjected to thin layer chromatography, GC/MS, and mass difference analysis to confirm resin loading.

Immobilization of Alkynes onto Epoxy Sepharose 6B Resin

Epoxy-activated 6B Sepharose (GE Healthcare, 200 mg) was added to a filter syringe and washed with distilled water (5 washes, 3 mL). Alkyn-ol (700 μmol) and coupling buffer (3.5 mL, pH 13.0) was added to a 15 mL tube followed by the resin. The mixture was shaken at room temperature for 16 h. The sepharose was transferred to a filter syringe and washed with coupling buffer (4mL). The sepharose was transferred to a 15 mL tube and capped with ethanolamine (3.5 mL). The sepharose was incubated at 30°C for four hours then washed in a filter syringe with acetate buffer (10 mM) and tris-HCl buffer (pH 4 and pH 8 respectively, 3 alternating washes with 3 mL each). Finally, the sepharose was left in acetate buffer with 20% ethanol to store it for further use.

General GFP Immobilization Conditions

An Eppendorf tube was charged with 5 mM Tris [(1-benzyl-1H-1, 2, 3-triazol-4-yl) methyl] amine (TBTA; 5 μL) along containing 4 mM tris (2-carboxyethyl) phosphine (TCEP; 5 μL) and 1 mM $CuSO_4$ (6 μL). The catalyst system was diluted in phosphate buffered saline solution (PBS; 15 μL buffer) and 2.3 mg/mL of GFP-*p*AzF mutant (15 μL) was added, followed by 20 mg of derivatized Sepharose 6B resin (GE Healthcare). This mixture was incubated at 4°C for 16 h then transferred to an empty spin column and washed with 1X PBS Buffer (5 x 200 μL) by centrifugation at 3,700 rpm for 60 s. The resultant resin was then analyzed using a Bio-Rad gel imager (BioRad Molecular Imager Gel Doc XR+) and relative fluorescence measurements were taken by transferring the resin samples to a 96-well plate on a plate reader (Biotek Synergy HT Microplate Reader).

Chapter 3: Progress towards the integration of unnatural amino acid immobilization methodology with a microwave system

3. A. Selection of a hyperthermophilic model system

While the methodology developed in the previous chapter does represent a powerful tool for expanding the utility of proteins in harsh chemical environments, it still suffers from long immobilization times and does little to address the expanded use of proteins in highly elevated temperatures. As examined earlier, microwave irradiation can be a powerful tool for decreasing reaction times and improving yields of organic reactions.[1] Thus, microwave heating could potentially increase the rate of protein immobilization and the rate of the reaction which the immobilized enzyme performs. However, microwave-assisted reactions can involve temperatures approaching 100 °C and beyond which are far beyond the typical denaturation temperatures of mesophilic enzymes (those coming from organisms which typically grow between 20 and 45 °C).[2] However, enzymes from hyperthermophilic microorganisms (those growing at and above 90 °C) typically only begin to display optimal activity around 90 °C and can maintain their activity in temperatures exceeding 100 °C.[2] For example, hyperthermophilic DNA polymerases are used daily in laboratories all around the world to amplify regions of DNA through the

polymerase chain reaction (PCR) which requires DNA denaturation steps of around 95 °C.[3] Thus, a system where a hyperthermophilic enzyme could be successfully incorporated into a microwave system was chosen as the next target for expanding the unnatural amino acid immobilization methodology developed above.

3. B. Use of hyperthermophilic enzymes in microwave environments and microwave-assisted PCR

Importantly for the proposed system incorporating microwave irradiation with hyperthermophilic enzyme catalysis, studies have shown that the activity of some hyperthermophilic enzymes can be increased by microwave heating relative to thermal heating due to the large dipole moment proteins typically contain.[4] In a study by Young et al. 2008, three hyperthermophilic enzymes were found to display significant increases in catalytic activity when exposed to microwave heating as compared to thermal heating even though the temperature maximum ultimately reached in both cases was far below the optimal temperature of most of these enzymes (See **Fig. 3.1**).[4] In addition, microwave-assisted PCR has also been reported in the literature.[5,6]
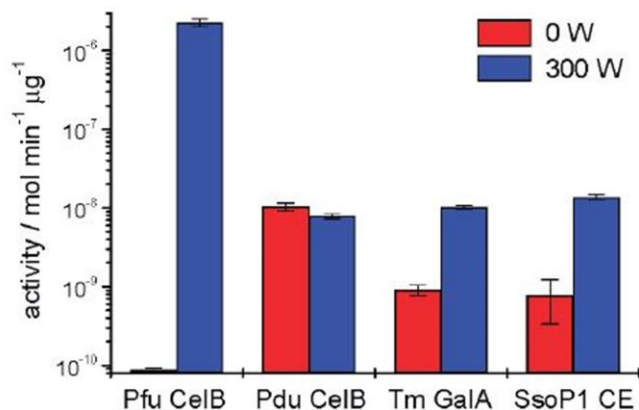
Figure 3.1. Activity of four hyperthermophilic enzymes (organism name followed by protein name) subjected to a thermal heating regimen (0 W of microwave power) and a microwave heating regimen (300 W). Both heating regimens reached approximately 40 °C. Adapted from *J. Am. Chem. Soc*. **2008**, *130*, 10048–10049.[3]

As with conventional thermal PCR, the microwave protocols used in both Fermer et al. 2003 and Orrling et al. 2004, both involved cycles of microwave irradiation designed to

mimic the cycles of a conventional thermal PCR.[5,6] These included an initial microwave pulse to denature DNA, then a cooling step to allow for the annealing of primers, followed by a second microwave pulse to activate the polymerase enzyme.[5,6] While neither of these papers could fully replicate the efficiency of conventional PCR, each microwave protocol had advantages in terms of shorter reaction times and higher reaction volumes (See **Fig. 3.2**).[5,6]

Unfortunately, all attempts to adapt these microwave protocols to our CEM microwave

Lane	Microwave power pulses	Template DNA	No. of cycles	Relative band intensity
1	100W/130W	Plasmid	25	0.13
2	100W/130W	Plasmid	35	0.38
3	Conventional PCR	Plasmid	25	1.0

Figure 3.2. Ethidium Bromide stained DNA gel displaying results of Fermer et al. microwave PCR protocols. M lanes contain DNA ladder. Adapted from *European Journal of Pharmaceutical Sciences*. **2003**, *18*, 129-132.[4]

system failed to produce any measurable amplification of DNA as analyzed on an ethidium bromide stained agarose gel (See **Table 3.1** for a general summary of Microwave PCR attempts and Experimental Section for detailed reaction schemes). It is possible that repeated exposure to microwave irradiation could denature the polymerase enzyme and that this effect is more pronounced on our newer model microwave as opposed to the older models used in the earlier studies. One future study that could be performed would be to assay the activity of the polymerase enzyme after each cycle to determine if it does indeed denature quickly upon exposure to repeated microwave irradiation.
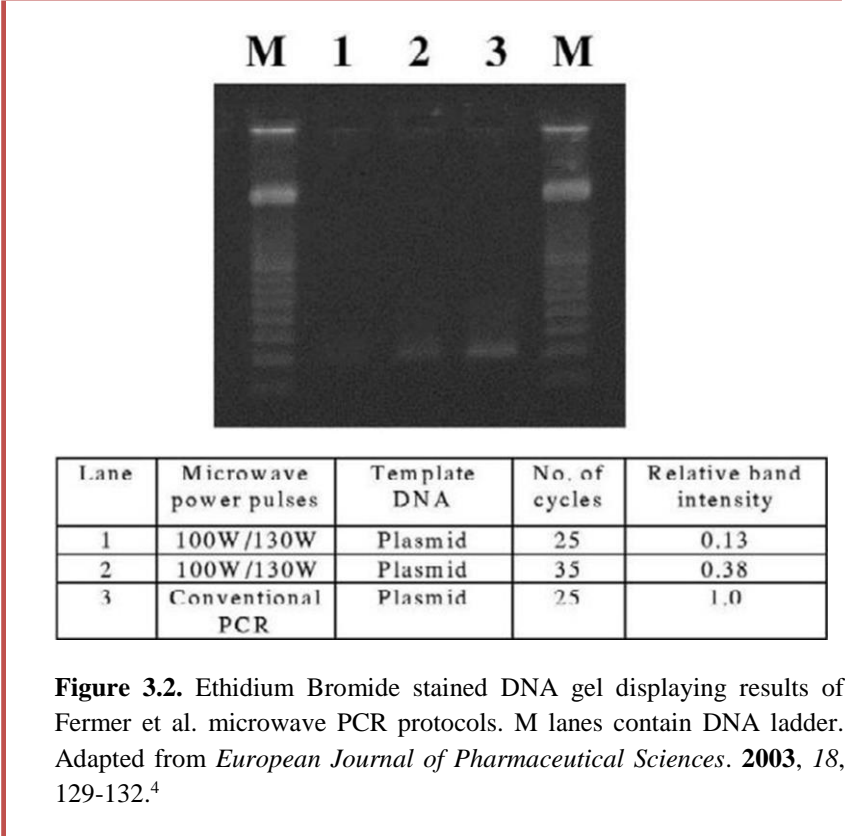
Micorwave PCR attempts							
Scheme #	Polymerase Used	Coolmate used (Y/N)	water baths used (Y/N)	mineral oil used (Y/N)	stir bar used (Y/N)	# of cycles	Reaction Volume (in μL)
1	Taq	N	Y	N	N	35	500
2	Taq	N	N	N	N	33	500
3	Taq	Y	N	N	N	40	500
4	Cheetah Taq	N	N	N	N	33	500
5	Cheetah Taq	N	N	N	N	33	500
6	Cheetah Taq	Y	N	N	N	40	500
7	Cheetah Taq	N	N	Y	Y	33	1000
8	Cheetah Taq	N	N	Y	N	33	1000
9	Cheetah Taq	N	N	N	Y	33	1000
10	Cheetah Taq	N	N	Y	Y	33	1000
11	KAPA Hotstart	N	N	N	N	33	1000
12	KAPA Hotstart	Y	N	N	N	40	1000
13	KAPA Hotstart	Y	N	N	N	40	1000
14	KAPA Hotstart	Y	N	N	N	60	1000
15	KAPA Hotstart	N	Y	Y	N	33	100
16	KAPA Hotstart	N	Y	Y	N	33	100

Table 3.1. General summary of microwave PCR attempts. For detailed schemes see Experimental Section.

Overall, it does appear that hyperthermophilic enzymes could potentially benefit from increased stability in microwave systems. In addition, most applications of hyperthermophilic enzymes also require aqueous buffers. Thus, the immobilization methodology developed in Chapter 2 could potentially increase the efficiency and applicability of microwave-assisted hyperthermophilic enzyme catalysis. In this proposed system, microwave heating could be used to increase the efficiency of the immobilization reaction of a hyperthermophilic enzyme onto a solid support. Then, the immobilized hyperthermophilic enzyme could potentially be used in organic solvents under microwave heating due to the increased stability afforded by immobilization onto the solid support and also recycled for multiple catalytic cycles due to the ease of separating the solid support from the reaction mixture. All in all, this proposed methodology holds a great deal of promise for further expanding the role of hyperthermophilic enzyme catalysis in both academic and industrial applications.

3. C. Selection of a hyperthermophilic model protein and whole-plasmid mutagenesis

To investigate this proposed methodology, a carboxylesterase from the hyperthermophilic organism *Sulfolobus solfataricus* (Sso) P1 was selected as a model protein.[7] This esterase (SsoEST) is approximately 33 kD and is able to cleave esters between aromatic groups and aliphatic carbon chains approximately 6-8 carbons in length.[7] An assay incorporating a *para*-nitrophenol ester has also been developed to study the activity of SsoEST.[7] When the enzyme cleaves this ester, it releases *para*-nitrophenol, which upon deprotonation displays characteristic absorbance at 405 nm and as a result can be used to measure the amount of enzyme activity.[7] (See **Fig 3.3)** A plasmid containing the Sso P1 esterase gene under the control of a lac operon and along with an ampicillin resistance gene was graciously obtained from the laboratory of Robert M. Kelly at North
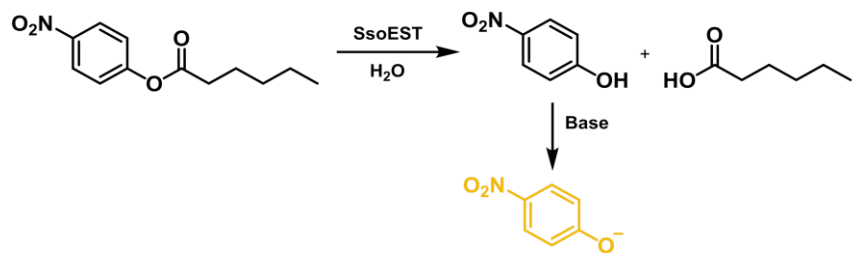
Fig 3.3. Scheme of *p*Np-hexanoate assay for measuring SsoEST activity.[7]

Carolina State University. The sequence of the esterase gene was provided by the Kelly lab and then used to create primers for site-directed whole plasmid mutagenesis. This was done in order to create plasmids with TAG stop codons at defined surface exposed residues (7, 28, 116, 184, and 235) of the protein. Site-directed mutagenesis of whole plasmids is a non-PCR thermocycling reaction that amplifies a whole plasmid (See **Fig 3.4**).[8, 9] Primers are designed to contain the desired mutation in the middle of a sequence that is otherwise perfectly complementary to the targeted plasmid. In addition, the forward and reverse

primers are designed so that DNA polymerization will proceed completely around the plasmid with the longer extension times used in mutagenesis protocols.[8] Then DpnI, a restriction enzyme that digests methylated DNA, is added to the reaction mixture. As the WT plasmid is typically obtained from a bacterial source, it will be endogenously methylated, whereas the recently synthesized mutated plasmid will be unmethylated.[8] Thus, DpnI digestion can remove the template DNA that does not contain the mutation and leave only the desired mutated plasmid, which is ready to use after DNA cleaning and concentrating steps.[8] Based on the specific annealing temperatures of the designed primers for the

Figure 3.4. Scheme of site-directed mutagenesis. Designed primers copy the whole plasmid and introduce a mutation. DpnI then digests the starting plasmid, leaving the mutated plasmid. Adapted from *Nature Methods*. **2007**, *4*, 455-461.[9]

Sso P1 EST gene, a site-directed mutagenesis protocol was developed and run for each of the five sets of primers in addition to one control reaction that did not contain any primers (See Experimental Section). After the DNA from each reaction was isolated and concentrated, the resulting plasmids were transformed into competent *E. coli* cells and grown on plates containing ampicillin. Mutant colonies were only collected when the control plate displayed no growth (If DpnI digestion was nearly complete then there would be almost no WT plasmids left in the control reaction which could impart ampicillin resistance to transformed cells). In this manner, colonies on each mutant plate were selected

and grown in culture to obtain plasmids for each set of primers. It should be noted however, the DpnI is not perfectly efficient and thus multiple colonies were selected for each mutant as some colonies might come from cells originally transformed with wild-type plasmid. To investigate whether the mutant plasmids truly had a TAG codon inserted into the desired position, the suspected 7TAG, 28TAG, and 116TAG plasmids were co-transformed into *E. coli* with the ambryx synthetase plasmid (See Experimental Section). Studies have not yet been conducted with the suspected 184TAG and 235TAG plasmids. These cells could then be grown up in culture and induced to produce both the Sso P1 EST and ambryx genes. Then, *p*AzF could be added to one expression culture of a certain plasmid and not to another identical one. If the investigated plasmid does contain a stop codon, then the +*p*AzF sample will be able to successfully suppress the stop codon and produce full length protein, whereas the −*p*AzF sample will not be able to suppress the stop codon and produce full length protein. As examined above however, the ambryx synthetase used was evolved to be more promiscuous than other orthogonal synthetases and thus can sometimes charge an orthogonal tRNA with a natural amino acid such as tyrosine. The ambryx-aaRS is more efficient at incorporating *p*AzF however and thus differential expression between +*p*AzF and −*p*AzF samples should be observed for plasmids containing TAG stop codons. If a cell instead does not contain a plasmid with a TAG codon, it will just express wild-type protein and there should be very little difference in protein expression between a +*p*AzF and a −*p*AzF sample. This differential expression of protein can then be measured on SDS-PAGE. For one 7TAG plasmid and one 28TAG plasmid, differential expression was observed between +pAzF and −pAzF samples (See **Fig 3.5**). Unfortunately, no differential

expression was observed for any 116TAG plasmid, suggesting that these samples were mostly likely residual wild type plasmids that survived DpnI digestion.
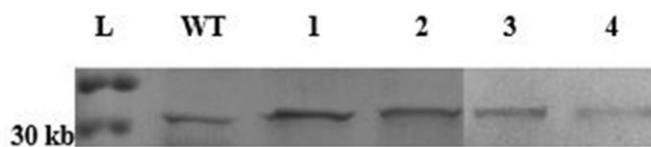
Figure 3.5. SDS-PAGE data from differential expression studies. L = DNA ladder, WT = wild-type SsoEST, 1 = Suspected SsoEST-28-TAG (+pAzF), 2 = Suspected SsoEST-28-TAG (-pAzF), 3 = Suspected SsoEST-7-TAG (+pAzF), 4 = Suspected SsoEST-7-TAG (-pAzF). Note: WT and SsoEST-7-TAG samples were less concentrated than SsoEST-28-TAG

3. D. Future studies and conclusions

While this project is not yet complete, progress has been made towards adapting the unnatural amino acid immobilization technology developed in Chapter 2 to a microwave system. In addition, these early studies have informed the future studies which remain to be completed. Early efforts to obtain SsoEST plasmids with mutations creating TAG stop codons at defined residues through site-directed mutagenesis have revealed that incomplete digestion of wild-type plasmid can be a lingering problem. Thus, studies will be conducted to optimize the DpnI digestion in addition to completing differential expression studies on suspected 116TAG, 184TAG, and 235TAG plasmids. In addition, promising mutant plasmids will be sequenced to confirm that they have the desired mutation in their nucleotide sequence at the correct position. Lastly, studies are currently underway to attach a fluorophore with an alkyne moiety to suspected SsoEST-7-pAzF and SsoEST-28-pAzF obtained from the differential expression studies as further confirmation

that these proteins do indeed have the unnatural amino acid inserted into the desired position.

Once all of the SsoEST mutants have been obtained, microwave studies can be conducted to evaluate the efficiency of this methodology. First, it must be determined whether Sepharose 6B resin is stable under microwave irradiation and whether there are any advantages, primarily with regards to reaction time, with performing the immobilization of the alkyne moiety on the solid support under microwave irradiation. Once the sepharose 6B resin (or another solid support) has been optimized for the microwave system, then studies can be conducted to see if SsoEST mutants containing *p*AzF at desired residues can be immobilized and retain their activity on the alkyne-derivatized support through the same cycloaddition used in Chapter 2 but under microwave irradiation. Conveniently, the activity of immobilized enzymes can be quickly analyzed using the absorbance assay developed by Kelly et al. 2003.[7] In these studies, it will first be important to determine whether the reported increases in SsoEST catalytic activity under microwave irradiation can be replicated with immobilized enzymes and also whether there is a bias in catalytic activity with regards to which residue the protein is immobilized through as was observed above for GFP. Then, it can be evaluated whether the microwave system can lead to reduced immobilization times and whether the immobilization process can stabilize the SsoEST enzyme so that it can be used with organic solvents and for multiple catalytic cycles.

This work represents the first step in trying to adapt the previously developed unnatural amino acid methodology to a microwave system utilizing hyperthermophilic enzymes. Microwave irradiation can lead to more efficient heating, increased reaction

rates, and also can increase certain enzymes' catalytic activity due to their typically high dipole moment. In addition, hyperthermophilic enzymes have remarkable stability and catalytic activity in elevated temperatures and have already been adopted for a multitude of industrial processes. Combining microwave heating with hyperthermophilic enzyme catalysis thus represents an exciting possibility for greatly improving the efficiency of a variety of organic reactions. However, hyperthermophilic enzymes still have reduced stability in organic solvents and, as learned from the studies with microwave PCR, they are potentially vulnerable to degradation after multiple cycles of microwave irradiation. Thus, protein immobilization through an unnatural amino acid represents a powerful and predictable way to potentially increase the stability of hyperthermophilic enzymes under microwave irradiation. In this way, some of nature's most hardy catalysts can hopefully be efficiently combined with microwave irradiation to greatly expand the utility of natural catalysis in industry, energy, and pure research.

Chapter 3 References

1. Herrero, M.; Kremsner, J.; Kappe, C. Nonthermal Microwave Effects Revisited: On the Importance of Internal Temperature Monitoring and Agitation in Microwave Chemistry. *J. Org. Chem*. **2008**, *73*, 36-47.
2. Adams, M.; Kelly, R. Finding and using hyperthermophilic enzymes. *Trends in Biotechnology*. **1998**, *16*, 329-332.
3. Gyllensten, U. PCR and DNA sequencing. *BioTechniques*. **1989**, *7*, 700-708.
4. Young, D.; Nichols, J.; Kelly, R.; Deiters, A. Microwave Activation of Enzymatic Catalysis. *J. Am. Chem. Soc*. **2008**, *130*, 10048–10049.
5. Fermer, C.; Nilsson, P.; Larhed, M. Microwave-assisted high-speed PCR. *European Journal of Pharmaceutical Sciences*. **2003**, *18*, 129-132.
6. Orrling, K.; Nilsson, P.; Gullberg, M.; Larhed, M. An efficient method to perform milliliter-scale PCR utilizing highly controlled microwave thermocycling. *Chem. Commun*. **2004**, *7*, 790-791.
7. Sehgal, A. C.; Callen, W.; Mathur, E.; Short, J. M.; Kelly, R. M. Carboxylesterase from *Sulfolobus solfataricus* P1. *Methods in Enzymology*. **2003**, *330,* 461-471.
8. Laible, M.; Boonrod, K. Homemade Site Directed Mutagenesis of Whole Plasmids. *Journal of Visualized Experiments*. **2009**, *27*, 1-3.

9. Smith, C. Cloning and mutagenesis: tinkering with the order of things. *Nature Methods*. **2007**, *4*, 455-461.

Experimental Section

Site-directed Mutagenesis protocol

To 6 clean 200 µL PCR tubes on ice, 27.5 µL of deionized water, 10 µL of KAPA Hifi Buffer, 5 µL of 2 mM dNTPs, 1 µL of 10 µM Fwd primer (7, 28, 116, 184, or 235), 1 µL of 10 µM Rev primer (7, 28, 116, 184, or 235), 2.5 µL of DMSO, 1 µL of KAPA Hifi polymerase and 2 µL of SsoEST plasmid were added. (Control reaction had no primers and 29.5 µL of deionized water) The tube was then mixed briefly and then added to the Bio-Rad thermocycler (model iCycler™ 96 well reaction module). The heating protocol follows below. Afterward, PCR samples were stored in the freezer.

	PCR heating protocol	
	Temperature (°C)	Time (min:sec)
Start	95	5:00
Cycling (x30)	98	0:20
	54	0:50
	72	7:00
End	72	7:00

DpnI digestion and DNA purification

To each PCR mixture, 2 µL of DpnI enzyme were added. These mixtures were then heated to 37 °C in the BioRad thermocycler for 2 hours. Afterwards, the reaction mixtures were added to Zymo-Spin™ DNA clean and concentrate columns. The plasmids were then purified according to the manufacturer's protocol and concentrated down to 10 µL solutions.

Transformation of mutagenesis plasmids into competent E. coli cells

Each plasmid obtained from the site-directed mutagenesis protocol (including control sample) was transformed into Escherichia coli BL21 (DE3) cells using an Eppendorf eporator electorporator. The cells were then plated and grown on LB agar in the presence

of ampicillin (50 mg/mL) at 37˚C overnight. If no colony growth was observed on the control plate, then 3 representative colonies from each mutant plate were used to inoculate LB media (4 mL) containing ampicillin. The cultures were incubated at 37˚C overnight and purified DNA was obtained from these colonies using a Zymo mini-prep kit according to the manufacturer's protocol.

Differential Expression Studies

A suspected SsoEST-TAG variant plasmid (0.5 μL) was co-transformed with a pEVOL-ambryx aaRS plasmid (0.5 μL) into *Escherichia coli* BL21 (DE3) cells using an Eppendorf eporator electorporator. The cells were then plated and grown on LB agar in the presence of chloramphenicol (34 mg/mL) and ampicillin (50 mg/mL) at 37˚C overnight. One colony was then used to inoculate LB media (4 mL) containing both ampicillin and chloramphenicol. The culture was incubated at 37˚C overnight and used to inoculate two expression cultures (10 mL LB media, 50 mg/mL Amp, 34 mg/mL Chlor) at an OD600 0.1. The cultures were incubated at 37˚C to an OD600 between 0.6 and 0.8 at 600 nm, and protein expression was induced by addition of 20 % arabinose (10 μL) and 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; 10 μL). To one culture for each SsoEST plasmid, *p*AzF (100 μL, 100 mM) was added while the matching culture received no *p*AzF. The cultures were allowed to shake at 30˚C for 16-20 h then centrifuged at 5,000 rpm for 10 minutes and stored at -80˚C for 3 hours. The cell pellet was re-suspended using 500 μL of Bugbuster (Novagen) containing lysozyme, and incubated at 37˚C for 20 minutes. The solution was transferred to an Eppendorf tube and centrifuged at 13,200 rpm for 10 minutes, then the supernatant was poured into a fresh Eppendorf tube which was then heated to 80 °C for 10 min to denature all non-thermophilic proteins. These samples were

then centrifuged again at 13,200 for 10 minutes and the supernatant was collected and concentrated down to obtain purified SsoEST. Purified SsoEST was then analyzed by SDS-PAGE (BioRad 10% precast gels, 150V, 1.5h), and stained gels were imaged by a BioRad image system to determine any differential expression between +/- pAzF samples.

Microwave PCR

Note: For each microwave PCR attempt, a conventional PCR was run in a BioRad iCycler 96 Well Reaction Module that approximately matched the reactant concentrations of the microwave attempt and under thermocycling conditions that approximately matched the microwave heating. In addition, as multiple closely related schemes were tried for microwave PCR, only a typical microwave protocol and typical conventional PCR protocol are written out below. Specific reactant amounts, and heating protocols for each scheme follow in the tables below.

Typical Conventional PCR Protocol

To a clean 200 μL PCR tube on ice, deionized water, Taq Buffer (or Cheetah Taq Buffer, or KAPA Hifi Buffer), 25 mM $MgCl_2$, 10 mM dNTPs, 10 μM Fwd aaRS primer, 10 μM Rev aaRS primer, Taq DNA polymerase (or Cheetah Taq polymerase, or KAPA Hifi polymerase), and a pEVOL DNA plasmid were added. The tube was then mixed briefly and then added to the Bio-Rad thermocycler and the heating protocol for the scheme being used was run directly. Afterward, PCR samples were stored in the freezer. For analysis of DNA amplification, 10 μL of the reaction mixture was mixed with 2 μL of DNA loading dye and run on an agarose gel stained with ethidium bromide in 1x TAE buffer for 25 min at 120 V.

Typical Microwave PCR Protocol

To a clean microwave vial on ice, deionized water, Taq Buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 10 μM Fwd aaRS primer, 10 μM Rev aaRS primer, Taq DNA polymerase (or Cheetah Taq DNA polymerase), and a pEVOL DNA plasmid were added. The amounts added to the microwave reaction were usually 10x the conventional PCR amounts (Some schemes 2x, or 20x). The tube was then mixed briefly and then added to the CEM microwave system or the CEM Coolmate system and the heating protocol for the scheme being used was run by pulsing the microwave at the desired temperatures or wattages with cooling steps in between. Some schemes also involved hot starts in water baths and annealing steps with water baths held at the annealing temperature. Afterward, PCR samples were stored in the freezer. For analysis of DNA amplification, 10 μL of the reaction mixture was mixed with 2 μL of DNA loading dye and run on an agarose gel stained with ethidium bromide in 1x TAE buffer for 25 min at 120 V.

Specific Microwave and Conventional PCR protocols

Scheme 1

Scheme 1						Conventional PCR heating protocol	
	Conventional PCR		Microwave PCR				
Reactant	(in μL)		(in μL)			Temperature (°C)	Time (min:sec)
DI water	37		370		Start	95	3:00
Taq Buffer	5		50		Cycling (x33)	95	0:20
25 mM MgCl2	3		30			54	0:30
10 mM dNTPs	1		10			68	1:00
10 μM Fwd primer	1		10		End	68	5:00
10 μM Rev primer	1		10				
Taq DNA polymerase	0.25		2.5				
pEVOL DNA plasmid	1.75		17.5				

Scheme 1 Microwave heating protocol

After addition of the reactants to the microwave vial, it was sealed and warmed in a water bath at 94 °C for 12 min. The reaction vessel was then equilibrated in a 54 °C water

bath for 1 min. Then, the reaction vessel was added to the microwave and pulsed with 100 W of microwave irradiation for 15 sec, followed by 30 sec of no irradiation, then pulsed with 130 W of microwave irradiation for 15 sec and finally equilibrated in a 54 °C water bath for 1 min. This cycle of pulses was then repeated 35x.

Scheme 2

Scheme 2						Conventional PCR	heating protocol
	Conventional PCR		Microwave PCR				
Reactant	(in µL)		(in µL)			Temperature (°C)	Time (min:sec)
DI water	37		370		Start	95	3:00
Taq Buffer	5		50		Cycling (x33)	95	0:20
25 mM MgCl2	3		30			54	0:30
10 mM dNTPs	1		10			68	1:00
10 µM Fwd primer	1		10		End	68	5:00
10 µM Rev primer	1		10				
Taq DNA polymerase	0.25		2.5			Microwave PCR	heating protocol
pEVOL DNA plasmid	1.75		17.5			Temperature (°C)	Time (min:sec)
					Start	93	3:00
					Cycling (x33)	94	0:20
						cool to 54	0:30
						68	1:00
					End	68	5:00

Scheme 3 Coolmate Microwave test

Scheme 3						Conventional PCR	heating protocol
	Conventional PCR		Microwave PCR				
Reactant	(in µL)		(in µL)			Temperature (°C)	Time (min:sec)
DI water	37		370		Start	95	3:00
Taq Buffer	5		50		Cycling (x33)	95	0:20
25 mM MgCl2	3		30			54	0:30
10 mM dNTPs	1		10			68	1:00
10 µM Fwd primer	1		10		End	68	5:00
10 µM Rev primer	1		10				
Taq DNA polymerase	0.25		2.5				
pEVOL DNA plasmid	1.75		17.5				

Scheme 3 Coolmate Microwave heating protocol

After addition of the reactants to the microwave vial, the reaction vessel was added to the Coolmate Microwave system which had been pre-cooled to -20 °C. The reaction vessel was then pulsed with 300 W of microwave irradiation for 20 sec or until it reached

25 °C which was the cut-off temperature. The sample was then allowed to cool back down again and then this process was repeated 40x.

Scheme 4 Cheetah Taq Polymerase

Scheme 4						
	Conventional PCR	Microwave PCR			Conventional PCR heating protocol	
Reactant	(in µL)	(in µL)			Temperature (°C)	Time (min:sec)
DI water	36	360		Start	95	3:00
Cheetah Taq Buffer	5	50		Cycling (x33)	95	0:20
25 mM MgCl2	3	30			54	0:30
10 mM dNTPs	1	10			68	1:00
10 µM Fwd primer	1	10		End	68	5:00
10 µM Rev primer	1	10				
Cheetah Taq polymerase	1	10			Microwave PCR heating protocol	
pEVOL DNA plasmid	2	20			Temperature (°C)	Time (min:sec)
				Start	93	3:00
				Cycling (x33)	85	0:20
					cool to 52	0:30
					63	1:00
				End	63	5:00

Scheme 5 Cheetah Taq Polymerase

Scheme 5						
	Conventional PCR	Microwave PCR			Conventional PCR heating protocol	
Reactant	(in µL)	(in µL)			Temperature (°C)	Time (min:sec)
DI water	36	360		Start	95	3:00
Cheetah Taq Buffer	5	50		Cycling (x33)	95	0:20
25 mM MgCl2	3	30			54	0:30
10 mM dNTPs	1	10			68	1:00
10 µM Fwd primer	1	10		End	68	5:00
10 µM Rev primer	1	10				
Cheetah Taq polymerase	1	10			Microwave PCR heating protocol (with stir bar)	
pEVOL DNA plasmid	2	20			Temperature (°C)	Time (min:sec)
				Start	93	3:00
				Cycling (x33)	93	0:20
					cool to 54	0:30
					66	1:00
				End	65	5:00

Scheme 6 Coolmate Microwave+Cheetah Taq Polymerase

Scheme 6						
	Conventional PCR	Microwave PCR			Conventional PCR heating protocol	
Reactant	(in µL)	(in µL)			Temperature (°C)	Time (min:sec)
DI water	36	360		Start	95	3:00
Cheetah Taq Buffer	5	50		Cycling (x33)	95	0:20
25 mM MgCl2	3	30			54	0:30
10 mM dNTPs	1	10			68	1:00
10 µM Fwd primer	1	10		End	68	5:00
10 µM Rev primer	1	10				
Cheetah Taq polymerase	1	10				
pEVOL DNA plasmid	2	20				

Scheme 6 Coolmate Microwave+Cheetah Taq Polymerase heating protocol

After addition of the reactants to the microwave vial, the reaction vessel was added to the Coolmate Microwave system which had been pre-cooled to -20 °C. The reaction vessel was then pulsed with 300 W of microwave irradiation for 20 sec or until it reached 25 °C which was the cut-off temperature. The sample was then allowed to cool back down again and then this process was repeated 40x.

Schemes 7-10 Cheetah Taq Polymerase (1 mL versions)

Scheme 7 – Reaction had 500 µL of mineral oil on top and a stir bar

Scheme 8 – Scheme 7 repeated without stir bar

Scheme 9 – Scheme 7 repeated with stir bar but no mineral oil

Scheme 10 – Scheme 7 repeated with temperatures 20 °C cooler

Scheme 7-10 (1 ml versions)						
	Conventional PCR	Microwave PCR			Conventional PCR heating protocol	
Reactant	(in µL)	(in µL)			Temperature (°C)	Time (min:sec)
DI water	36	727		Start	95	3:00
Cheetah Taq Buffer	5	100		Cycling (x33)	95	0:20
25 mM MgCl2	3	60			54	0:30
10 mM dNTPs	1	60			68	1:00
10 µM Fwd primer	1	4		End	68	5:00
10 µM Rev primer	1	4				
Cheetah Taq polymerase	1	5			Microwave PCR heating protocol	
pEVOL DNA plasmid	2	40			Temperature (°C)	Time (min:sec)
				Start	88	3:00
				Cycling (x33)	88	0:15
					cool to 52	0:40
					60	1:00
				End	60	5:00

Scheme 11 KAPA Hotstart Polymerase (1 mL)

Scheme 11					Conventional PCR heating protocol	
	Conventional PCR	Microwave PCR				
Reactant	(in µL)	(in µL)			Temperature (°C)	Time (min:sec)
DI water	36	703		Start	95	3:00
5x KAPA Hifidelity buffer	5	200		Cycling (x33)	95	0:20
25 mM MgCl2	3	36			54	0:30
10 mM dNTPs	1	30			68	1:00
10 µM Fwd primer	1	4		End	68	5:00
10 µM Rev primer	1	4				
KAPA Hotstart polymerase	1	3			Microwave PCR heating protocol	
pEVOL DNA plasmid	2	20			Temperature (°C)	Time (min:sec)
				Start	88	3:00
				Cycling (x33)	88	0:15
					cool to 52	0:40
					60	1:00
				End	60	5:00

Schemes 12-14 Coolmate Microwave+KAPA Hotstart Polymerase (1mL)

Schemes 12-14					Conventional PCR heating protocol	
	Conventional PCR	Microwave PCR				
Reactant	(in µL)	(in µL)			Temperature (°C)	Time (min:sec)
DI water	36	703		Start	95	3:00
5x KAPA Hifidelity buffer	5	200		Cycling (x33)	95	0:20
25 mM MgCl2	3	36			54	0:30
10 mM dNTPs	1	30			68	1:00
10 µM Fwd primer	1	4		End	68	5:00
10 µM Rev primer	1	4				
KAPA Hotstart polymerase	1	3				
pEVOL DNA plasmid	2	20				

Scheme 12 Coolmate Microwave+KAPA Hotstart Polymerase heating protocol

After addition of the reactants to the microwave vial, the reaction vessel was added to the Coolmate Microwave system which had been pre-cooled to -20 °C. The reaction vessel was then pulsed with 200 W of microwave irradiation for 15 sec or until it reached 25 °C which was the cut-off temperature. The sample was then allowed to cool back down again and then this process was repeated 40x. At pulses 20, 25, 30, and 35 aliquots were taken out for analysis by gel electrophoresis.

Scheme 13 Coolmate Microwave+KAPA Hotstart Polymerase heating protocol

After addition of the reactants to the microwave vial, the reaction vessel was added to the Coolmate Microwave system which had been pre-cooled to -20 °C. The reaction

vessel was then pulsed with 50 W of microwave irradiation for 60 sec or until it reached 25 °C which was the cut-off temperature. The sample was then allowed to cool back down again and then this process was repeated 40x.

Scheme 14 Coolmate Microwave+KAPA Hotstart Polymerase heating protocol

After addition of the reactants to the microwave vial, the reaction vessel was added to the Coolmate Microwave system which had been pre-cooled to -20 °C. The reaction vessel was then pulsed with 100 W of microwave irradiation for 30 sec or until it reached 25 °C which was the cut-off temperature. After cooling, the reaction vessel was then alternately pulsed with 130 W of microwave irradiation for 30 sec or until it reached 25 °C which was the cut-off temperature. The sample was then allowed to cool back down again and then this process was repeated 60x.

Schemes 15-16 KAPA Hotstart Polymerase (100 μL)

These reactions had 150 μL of mineral oil on top

Schemes 15-16					Conventional PCR heating protocol	
	Conventional PCR	Microwave PCR				
Reactant	(in μL)	(in μL)			Temperature (°C)	Time (min:sec)
DI water	36	66		Start	95	3:00
5x KAPA Hifidelity buffer	5	20		Cycling (x33)	95	0:15
25 mM MgCl2	3	0			54	0:40
10 mM dNTPs	1	3			68	1:00
10 μM Fwd primer	1	3		End	68	5:00
10 μM Rev primer	1	3				
KAPA Hotstart polymerase	1	1				
pEVOL DNA plasmid	2	4				

Scheme 15 Microwave heating protocol

After addition of the reactants to the microwave vial, it was sealed and warmed in a water bath at 94 °C for 12 min. Then, the reaction vessel was added to the microwave and pulsed with 100 W of microwave irradiation for 15 sec, followed by 30 sec of no irradiation, then pulsed with 130 W of microwave irradiation for 15 sec and finally equilibrated in a 54 °C water bath for 1 min. This cycle of pulses was then repeated 33x.

Scheme 16 Microwave heating protocol

After addition of the reactants to the microwave vial, it was sealed and warmed in a water bath at 94 °C for 12 min. Then, the reaction vessel was added to the microwave and heated to 70 °C before being equilibrated in a 54 °C water bath for 1 min. This cycle of pulses was then repeated 33x.