2014-12-01

Enhancing Protein and Enzyme Stability Through Rationally Engineered Site-Specific Immobilization Utilizing Non-Canonical Amino Acids

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A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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December 2014

ABSTRACT

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The demand for economical, efficient protein production, reuse, and recovery has never been greater due to their versatility in a large variety of applications ranging from industrial chemical manufacturing to pharmaceutical drug production. The applications for naturally and artificially produced proteins include protein drugs and other pharmaceutical products, as biocatalysts in environmentally friendly chemical manufacturing, as enzymes for food processing purposes, and as an essential component in many biomedical devices. However, protein production suffers from many challenges, which include the cost of production, protein stability especially under harsh conditions, and recoverability and reusability of the proteins. The combination of two developing technologies, cell-free protein synthesis systems (CFPS) and unnatural amino acid incorporation, provides solutions to these protein production challenges.

This dissertation reports on the use of cell-free protein synthesis systems and unnatural amino acid incorporation to develop new proteins and enzyme immobilization techniques that significantly increase activity and stability while simplifying recoverability and reuse.

Keywords: Synthetic biology, biocatalysis, enzyme immobilization, cell-free synthetic biology, green manufacturing, protein immobilization, click chemistry.

ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor Dr. Bundy for his guidance and support throughout my research. I thank my wonderful graduate committee members, Dr. Griffitts, Dr. Robison, Dr. McCleary, and Dr. Lewis for their wise counsel and support for me throughout my time here at BYU. I cannot thank them enough for the many lessons and great advice that I have received from their example.

I am also grateful for the many Bundy lab researchers who have significantly contributed to my research including Mark Smith, Mark Lindsay, Chad Varner, Derek Bush, Chris Hutchings, Steven Stanley, Garrett Laugenour, Dan Barfuss, Amin Salehi, Andrew Broadbent, Matt Schinn, and many others. Ryan VanYperen and Phil Bennellack of the Griffitts lab also helped to provide great counsel for my research.

I would like to acknowledge my family and friends for their support throughout my time here at BYU. They have definitely helped me to always remember the bigger picture.

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1. Introduction

1.1 Background

The demand for economical, efficient protein production, reuse, and recovery has never been greater due to their versatility in a large variety of applications ranging from industrial chemical manufacturing to pharmaceutical drug production (Clark, 2009). The applications for naturally and artificially produced proteins include protein drugs and other pharmaceutical products, as biocatalysts in environmentally friendly chemical manufacturing, as enzymes for food processing purposes, and as an essential component in many biomedical devices (Alcalde et al., 2006; Schmid et al., 2001). However, protein production suffers from many challenges, which include the cost of production, protein stability especially under harsh conditions, and recoverability and reusability of the proteins. The combination of two developing technologies, cell-free protein synthesis systems (CFPS) and unnatural amino acid incorporation, provides solutions to these protein production challenges.

Cell-free protein synthesis systems or in vitro protein expression systems enable unparalleled access to the protein production environment when compared to in vivo systems (Carlson et al., 2011). This open access allows for careful optimization and control of such environmental conditions such as pH, salt concentrations, redox potential, energy sources, mRNA, addition of catalysts and chaperones, and many others (Ahn et al., 2008b; Park et al., 2011a; Park et al., 2009c; Purnick and Weiss, 2009). Cell metabolism is focused primarily on survival and reproduction. The elimination of this evolutionary imperative enables the manipulation of cell metabolic systems to focus entirely on the production of a target protein or set of proteins (Ranjil et al., 2013). Such manipulation of the in vivo environment facilitates production of proteins that may be difficult or impossible to produce in large quantities in vivo

such as cytotoxic proteins or proteins containing unnatural amino acids or artificial moieties (Kigawa et al., 2002; Ranjil et al., 2013).

Of the many unique proteins that the direct access of the cell-free system enables, proteins incorporating non-canonical amino acids with useful functional groups are particularly versatile for applications ranging from bioimaging, vaccines, drug production, and biocatalysis. Non-canonical amino acids may include photoactivatable cross-linkers, fluorescent functional groups; metal binding functional groups; and many other groups containing a large variety of versatile and valuable qualities enabling the creation of new proteins and enzymes with never before created traits.

Given the versatility of the synthetic biology cell-free protein production system and its growing abilities to produce new and completely unique proteins that cannot be naturally produced, this dissertation reports on research advancements made in the course of developing the first truly covalent protein and enzyme immobilization technology that enables immobilization at potentially any residue using bioorthogonal and biocompatible techniques. Research advancements made while developing this technology include advancements in the producing proteins with non-canonical amino acids, improved understanding of the effects of non-canonical amino acid incorporation on protein activity and stability, and the major improvements in protein and enzyme activity and stability when immobilized in a rationally directed and site-specific manner. For the ease of the reader, a guide to the chapters is provided below.

1.1 Guide to the Chapters

A significant proportion of this dissertation consists of adaptations from publications that I personally led, developed, and authored. As described on a chapter by chapter basis, other

researchers provided valuable and significant contributions to this work. These chapters are summarized below.

Chapter 2: Transforming Synthetic Biology with Cell-free Systems.

This chapter is an adaptation from a review chapter from Synthetic Biology: Tools and Applications published in June 2013. Although the review chapter was written in collaboration with the Jewett Research Group at Northwestern, it has been edited to remove all sections written by our collaborators and includes only sections written by me with valuable support from my advisor, Bradley C. Bundy.

Chapter 3: Enhanced Protein Stability Through Minimally Invasive, Direct, Covalent, and Site-specific Immobilization

This chapter is an adaptation from an article entitled "Enhanced Protein Stability Through Minimally Invasive, Direct, Covalent, and Site-specific Immobilization" published in Biotechnology Progress in January 2013. This chapter discusses the major stability benefits from site-specifically and covalently immobilizing sfGFP onto a superparamagnetic bead surface.

Chapter 4: Enhancing Candida antarctica Lipase B Activity, Stability, and Reusability Through Rationally Directed, Site-specific Immobilization

This chapter discusses adaptations of the E. coli-based prokaryotic cell-free protein synthesis system to produce the eukaryotic, industrially relevant enzyme Candida antarctica Lipase B with non-canonical amino acids.

Chapter 5: Enhanced Enzyme Stability Through Site-Directed Covalent Immobilization

This chapter discusses the introduction of the first truly covalent, site-specific enzyme immobilization technique that enables immobilization at potentially any residue throughout the enzyme. The effects of enzyme stability predictions from previous molecular modeling studies

are compared with experimental data produced using our new technology. Significant improvements in enzyme stability over traditional techniques are demonstrated under harsh, denaturing conditions.

2. Transforming Synthetic Biology with Cell-Free Systems

This chapter is an adaptation from a review chapter titled "Transforming Synthetic Biology with Cell-free Systems" in Synthetic Biology: Tools and Applications published in June 2013. Although the chapter was written by me with substantial assistance from my advisor in collaboration with the Jewett Research Group of Northwestern University, it has been edited to remove all sections written by our collaborators.

2.1 Introduction

Synthetic biology is a new approach for engineering biology by design. At its heart, this emerging discipline seeks to make biology easier to engineer and to harness biology to serve society. Already, this new paradigm for engineering biology is enabling a deeper understanding of living systems and opening the way to sustainable and renewable energy production, cost-effective and widely accessible programmable medicines, and new solutions for environmental stewardship. While most synthetic biology efforts have focused on engineering living organisms, the cell's operating system and endogenous pathways pose an obstacle to synthetic biology efforts. This is because the cell's evolutionarily optimized agenda for growth and adaptation are often at odds with the engineer's objectives. Additionally, the complexity of living cells is still beyond our comprehension, and serves as a disadvantage to using living cells to develop synthetic biology applications. Cell-free systems circumvent these obstacles, and are emerging as a powerful approach for harnessing and expanding the capabilities of natural biological systems.

Over the last several years there has been tremendous development and innovation in the growing, yet still small, field of cell-free synthetic biology. This chapter focuses on recent developments and current applications in this field. We begin by defining cell-free biology and its advantages. We then highlight existing applications in cell-free synthetic biology from

proteins to metabolites (Figure 2-1). Finally, we consider some challenges and opportunities necessary to propel the field forward.

2.2 Cell-free Biology

Cell-free biology is the activation of complex biological processes without using intact, living cells. Bypassing cell walls, this approach opens up a whole new biological world, one without the need or ability to preserve DNA heritage. Moreover, this approach enables one to access and manipulate biology directly. In contrast to in vivo engineering efforts, this direct relationship with biocatalytic enzymes provides the ability to focus metabolism on the production of a single compound and removes physical barriers (allowing easy substrate addition, product removal, and rapid sampling). Furthermore, avoiding competition between cell growth (catalyst synthesis) and production (catalyst utilization) through the use of cell-free systems provides efficiency benefits for how we use synthetic biology for synthetic chemistry. In short, cell-free systems provide an unprecedented freedom of design to modify and control biological systems.

Cell-free systems can be generated from crude extracts or purified components. Crude extracts are prepared by lysing cells (typically harvested in the exponential growth phase) and are cheaper, as well as simpler to produce than purified systems. While crude extract-based systems are prominent in the field and have now been commercialized, purified systems also have their merits (Zawada et al., 2011). Specifically, the fact that every component of a purified system is well-characterized and can be tuned provides advantages for engineering design. Furthermore, purified systems lack unwanted activities (such as proteases and nucleases) that might be deleterious to crude extract systems. An example of a well-utilized purified system is the PURE system for cell-free translation, pioneered by the Ueda group (Shimizu et al., 2001).

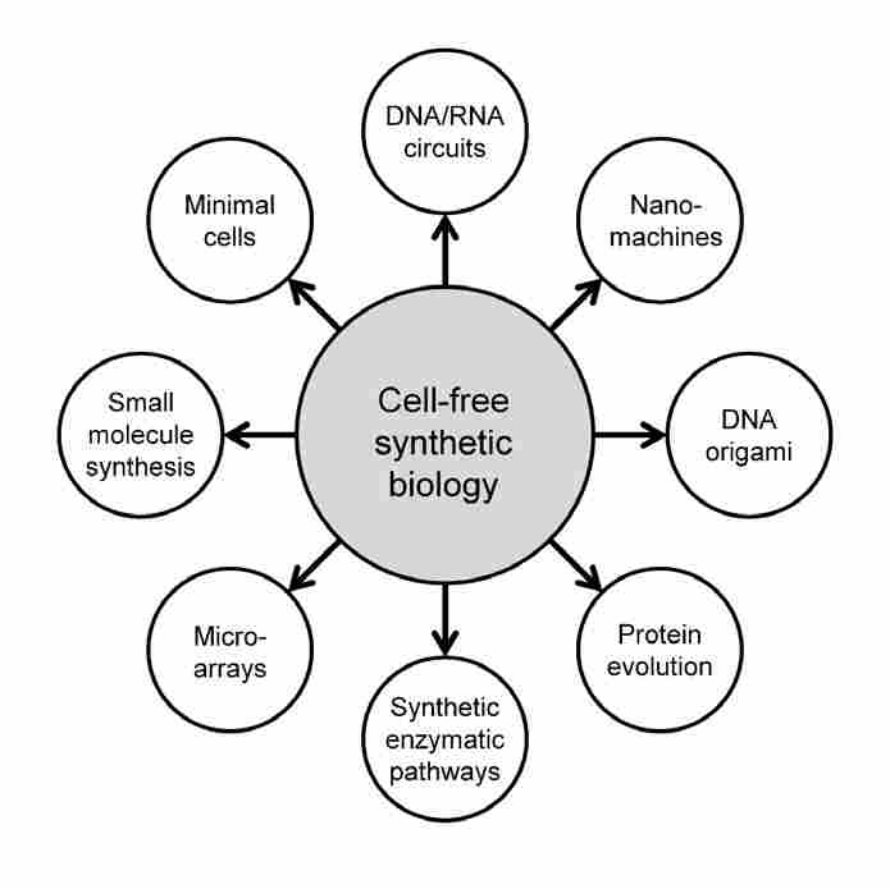


Figure 2-1: Applications of Cell-free Synthetic Biology (Ranjil et al., 2013).

Scale has long been considered a technological limitation of cell-free systems. Due to recent advances in cost-effective high-level protein synthesis (Carlson et al., 2011), E. coli-based cell-free protein synthesis systems have been successfully scaled-up to the manufacturing scale (Zawada et al., 2011). Production rates, production yields, and product activity were maintained over a volumetric expansion of 6 orders of magnitude (Voloshin and Swartz, 2005; Zawada et al., 2011). This pioneering advance has transformed cell-free systems from a foundational bench-top tool into an enabling technology with promise for commercial scale applications.

2.3 Advantages of Cell-free Biology

Cell-free technologies simplify biological systems and remove unnecessary overheads for engineering efforts. In this section, we highlight some of the many advantages gained by

removing physical barriers and decoupling cellular catalysts from the genetic architecture of the cell.

Direct Environment Control of an Open System

Cell-free biology enables direct access to and control of the biological environment. In living cells, the organism must maintain an environment conducive to every biocatalytic mechanism necessary for survival. In contrast, the cell-free environment can be directly optimized for a user-defined objective. The indispensable microbiology workhorse of polymerase chain reaction (PCR) is a simple but powerful cell-free synthetic biology example where the DNA polymerase, template, primer, and salt concentrations are directly controlled and optimized in a nuclease-free environment (Saiki et al., 1988). In another useful but simple cell-free example, RNA transcription can also be directly optimized while maintaining an RNase-free environment (Sousa and Mukherjee, 2003). In cell-free protein synthesis, the capability to directly control variables such as ionic strength, pH, redox potential, hydrophobicity, and enzyme and reactant concentrations has provided extraordinary advantages.

Direct Influence of Reaction Networks

Open cell-free biology enables the addition or removal of catalysts and/or reagents to directly influence reaction networks. In one example, Zhang and coworkers have demonstrated the optimized combination of 12 enzymes for 99.6% energy-efficient biohydrogenation of xylose and cellobiose to xylitol (Wang et al., 2011). This example, along with others like it, showcases the freedom of design for building metabolic networks from the ground up using cell-free systems (Jewett et al., 2008; Zhang et al., 2007a; Zhang et al., 2011b). In another example, cell-free circuits can be built and controlled by the addition of enzymes to the nucleic-acid-based circuits to control their rates of synthesis and degradation (Kim et al., 2006b). In the case of cell-

free protein synthesis, the open environment enables simplified preparations of PCR products for protein expression. Expression templates can be directly added and the concentration controlled for optimization. In one example, linear expression templates (LETs) prepared using PCR have been effectively used in cell-free translation systems by multiple research groups (Lesley et al., 1991; Park et al., 2011a; Woodrow et al., 2006). LETs obviate the need for time-consuming gene-cloning steps, accelerating process and product development pipelines. The ability to use LETs in the cell-free system is particularly valuable when the goal is the production of a large array of gene products, such as in genomic studies (Woodrow et al., 2006).

Direct Product Access

In cell-free systems, the lack of cell walls or membranes facilitates online product monitoring, one-step purification and recovery of DNA, RNA, or protein (Ahn et al., 2008a; Alimov et al., 2000). In the case of cell-free protein synthesis, several single-step purification processes using affinity chromatography have been demonstrated (Alimov et al., 2000; Jewett and Swartz, 2004b). These include strep- and his-tag purification and in situ product purification using magnetic affinity beads (Kim et al., 2006d). In another illustrative model, Bujara et al. coupled cell-free metabolic engineering to mass spectrometry analysis for direct profiling of rate-limiting steps to optimize multienzyme catalysis of dihydroxyacetone phosphate (DHAP) from glucose (Bujara et al., 2010).

Focusing Biological Machinery on a Single User-Defined Objective

Without the need, or ability, to support ancillary processes required for adaptation and growth, cell-free systems offer the ability to focus metabolism towards the exclusive objective of the engineer, and not the cell. This affords efficiency advantages necessary for producing complex biochemical products.

Accelerating Design-Build-Test Cycles

Engineering biology today is a time- and money-intensive process. Therefore, one of the key objectives of synthetic biology is to accelerate the design-build-test loops required for engineering biology. Right now, in vivo approaches take, on average, 3-4 months to complete this cycle (Dr. Alicia Jackson, DARPA 'Living Foundries' Industry Day 2011, personal communication). Much of this time is taken to identify and modify potential genes, and assemble and synthesize the potential pathways in living organisms. However, the design cycle limit of in vivo synthetic biology projects could at best potentially scale (in the future) as the growth rate of the organism and time for transformation. In cell-free systems, the design cycle is not limited by how fast cells reproduce. Rather, it can be faster, potentially approaching the limit of synthesizing the components (DNA, RNA, proteins). Ultimately, cell-free systems may therefore speed up the design cycle for engineering by more than 10-fold relative to in vivo approaches, and could be used as their own biomanufacturing platforms, or as feedback in the design of in vivo platforms.

Decreased Effects of Toxicity

Due to cell death and hindered cell growth, in vivo production of both cytotoxic products and products synthesized from cytotoxic substrates is a challenge. Unhindered by cell viability constraints, cell-free systems address this challenge. Over the last decade, researchers have demonstrated the use of cell-free systems to produce a growing number of cytotoxic products (Katzen et al., 2005). These include proteins with cytotoxic amino acids (Kigawa et al., 2002; Usón and Sheldrick, 1999), cytolethal disease toxins (Avenaud et al., 2004), cytotoxic proteins (Smith et al., 2011), and others (Wuu and Swartz, 2008). In one exemplary approach, hepatitis researchers studying a cytolethal distending toxin from Helicobacter hepaticus had been unable

to produce sufficient levels of protein in vivo to observe its mechanism of action due to its high cytotoxicity. Using a cell-free approach, researchers were able to produce sufficient quantities to examine the toxin's effect on the liver (Avenaud et al., 2004). In another illustrative case, cell-free translation of the cytotoxic A2 protein led to yields almost 1000 times higher than previously reported with in vivo production (Smith et al., 2011).

Expanding the Chemistry of Life

The products of biological systems are governed by the chemistry of life, which is limited to natural building blocks. Cell-free systems offer advantages for using synthetic biology for nonstandard chemistry (Harris and Jewett, 2012). One of the most prominent examples is using cell-free protein synthesis for site-specific incorporation of non-natural amino acids. In contrast to in vivo systems, there are no transport limitations for getting non-natural amino acids into the cell, and there is flexibility for reprogramming the genetic code because cellular viability need not be maintained. By employing cell-free protein synthesis, the putatively transport-limited p-propargyloxyphenylalanine was incorporated site-specifically into proteins at significantly higher production yields than in vivo systems (Bundy and Swartz, 2010). The high-yielding site-specific incorporation of azidophenylalanine and the global replacement of methionine by an unnatural analogue have also been demonstrated with cell-free systems (Goerke and Swartz, 2008; Patel and Swartz, 2011).

2.4 Utilizing Cell-free Systems for Protein Production

In contrast to in vitro efforts in DNA and RNA that mainly center on building and understanding nucleic acid circuitry, major in vitro efforts in proteins have been focused on synthesis and evolution strategies. Indeed, a technological renaissance has reinvigorated cell-free protein synthesis (CFPS) technologies over the past decade (Carlson et al., 2011). This

progress has realized protein yields exceeding grams of protein produced per liter reaction volume, cost reductions of multiple orders of magnitude, and microscale to manufacturing scale production. Here we discuss both bottom-up and top-down approaches to CFPS, along with frontier applications enabled by recent advances.

Bottom-up Cell-free Protein Synthesis Systems

The bottom-up approach to CFPS centers on using purified components. Pioneered by Ueda and colleagues, the most prevalent bottom-up system is the PURE system (Shimizu et al., 2001; Shimizu et al., 2005). In the PURE system, cellular machinery necessary for translation is independently overexpressed, purified, and combined in a test tube (Ohashi et al., 2010). An advantage of the PURE system is that the researcher is afforded the greatest management of every aspect of the protein synthesis process. Indeed, the ability to mix and match nearly any component of translation has proven remarkably useful for efforts to fold proteins and to incorporate non-natural amino acids (Harris and Jewett, 2012; Shimizu et al., 2005). The main disadvantage to this system is its cost. The necessity of expressing, purifying, and adding each component greatly increases the reagent cost and time required compared to top-down systems (Hodgman and Jewett). A cost comparison of the CFPS systems discussed herein is provided as Table 2-1.

Top-down Cell-free Protein Synthesis Systems

The top-down approach involves the modification and engineering of crude cell extract for protein production. Although any organism can potentially provide a source of crude lysate, the most frequently utilized crude extract systems are made from Escherichia coli, wheat germ, rabbit reticulocytes, and insect cells (Carlson et al., 2011).

The E. coli-based CFPS systems are currently the most widely used due to lower labor

requirements for cell growth and extract preparation, lower-cost energy sources, higher yields, greater rate of protein synthesis, and commercial scalability (Swartz, 2006; Zawada et al., 2011). Given the significant number of proteins previously expressed in this system and its high linear scalability (from 15 μL to 100 liters), the E. coli-based CFPS currently demonstrates the greatest versatility and economic efficiency (Table 2-1) (Carlson et al., 2011). However, this cell-free system still faces post-translational modification challenges, although a new report has described glycosylation in these systems for the first time(Guarino and DeLisa, 2012). Other bacteria-based cell-free systems have been developed, notably from thermophiles such as Thermus thermophiles (Uzawa et al., 2002).

Eukaryote-based CFPS systems are mainly derived from wheat germ, rabbit reticulocytes, and insect cells. Wheat germ extract-based cell-free systems provide the highest yields of the major eukaryotic cell-free systems (Madin et al., 2000; Park et al., 2009c). Using a cell-free protein-producing gel, one group reported yields as high as 5 mg/ml, approaching the highest yields reported for E. coli-based cell-free synthesis, but at much lower volumes (Park et al., 2009c). Unfortunately, wheat germ extract preparation is more costly and more labor-intensive than E. coli-based systems. Rabbit reticulocyte-based cell-free systems require a highly technical and difficult extract preparation method, but are used because of their ability to enable some post-translational modifications (Hancock, 1995; Pelham and Jackson, 1976; Ryabova et al., 1989). Low expression yields linked to the significant amount of ribonuclease M in the lysate, however, limit their utility (Endo and Sawasaki, 2006b). Additionally, the collection of sufficient amounts of reticulocytes requires harmful chemical treatments of rabbits (Ryabova et al., 1989).

Table 2-1: Common Energy Sources and the Relative Cost of Cell-free Protein Production Using Commercial Kits (Ranjil et al., 2013).

CFPS Type	Commonly Used Energy Sources	Extract Preparation Time	Relative Cost (per ng protein yield)*
Escherichia coli Lysate	• Phosphoenolpyruvate (Jewett and Swartz, 2004a) • Pyruvate (Jewett and Swartz, 2004a) • Acetyl kinase/acetyl phosphate (Ryabova et al., 1995) • Glucose (Calhoun and Swartz, 2005) • Creatine kinase/creatine phosphate (Park et al., 2011a) • Creatine kinase/creatine phosphate/glucose (Kim et al., 2007) • Creatine kinase/creatine phosphate/cAMP (Ma et al., 2010)	1-2 days	~$0.70-$1**(Endo and Sawasaki, 2006a)
Wheat Germ Lysate	• Creatine kinase/creatine phosphate (Madin et al., 2000)	4-5 days	~$1.50-$3*** (Carlson et al., 2011)
Insect Cell Lysate	• Creatine kinase/creatine phosphate (Ezure et al., 2006b)	1-2 days	~$4-$9.25****
Purified Component	• Creatine kinase/creatine phosphate (Ueda et al., 2010)	Extract not used, 32 individually purified components.	~$11******
Rabbit Reticulocyte Cell Lysate	• Creatine kinase/creatine phosphate (Hino et al., 2008)	4 days for rabbit treatment, 1 day for extract prep	~$30-$100***** (Endo and Sawasaki, 2006a)

* Cost assessed using maximum yields of respective commercial kits.
** Expressway Maxi Cell-free E. coli Expression System from Invitrogen, S30 T7 High-yield Expression Kit from Promega,
*** Wheat Germ Extract Cell Free Protein Synthesis Kits from Genecopoeia,
**** TnT® T7 Insect Cell Extract Protein Expression System from Promega
***** TnT-coupled Reticulocyte Lysate System from Promega
****** PURExpress In vitro Protein Synthesis Kit from NEB.

Commonly produced from Spodoptera frugiperda cells, insect-based CFPS provide an

effective combination of ample protein yield and post-translational modifications (Ezure et al., 2006a). Commercial insect cell-based systems have reached yields of up to 50 μg/ml (Ezure et al., 2006a). They also have been used successfully for several forms of post-translational modifications including glycosylation, isoprenylation, acetylation, N-myristoylation, and others (Suzuki et al., 2006, 2007; Tarui et al., 2001). Due to the cost of insect cell cultivation and extract preparation, insect cell-based cell-free systems are more costly than E. coli-based or wheat germ-based systems. While each of the CFPS systems developed to date has their merits and trade-offs, we turn our attention to several case studies that highlight the advantages of having direct access and control to the reaction conditions, as well as emerging applications made possible by recent technical advances.

2.5 Advantages Achieved By Directly Influencing Reaction Conditions

As mentioned above, cell-free biology provides the ability to directly manipulate and modulate reaction conditions. We now highlight several illustrative examples where ionic strength, pH, redox potential, hydrophobicity, and translation components were tuned. The overall ionic strength and the relative concentration of a specific ion can significantly impact the structure and activity of many enzymes and proteins (Hu et al., 2011; Jaenicke, 1991; Sedov et al., 2011; te Velthuis et al., 2010). For example, macromolecular protein structures such as virus-like particles have been shown to assemble at higher efficiencies as the ionic strength is increased (Zlotnick et al., 2002). Viral RNA polymerases are commonly inhibited by high concentrations of zinc (te Velthuis et al., 2010). Cell-free biology enables direct optimization of the ionic strengths for both the performance of the enzyme machinery and the activity of the product protein. By utilizing cell-free translation and assembly reactions, the highly efficient translation and assembly of macromolecular virus capsids has been demonstrated (Bundy et al.,

2008; Bundy and Swartz, 2011; Smith et al., 2011). Also, the cell-free assembly of the human hepatitis B virus and human papillomavirus capsids were observed to be the highest at ionic strengths that would be cytotoxic to most cells (Ceres and Zlotnick, 2002; Zlotnick et al., 2002).

Cell-free biotechnology also enables the ability to directly control the redox potential, which is not possible in cells. In an exemplary illustration, Zawada et al. carried out a combinatorial optimization of reaction conditions for both protein expression and folding (Zawada et al., 2011). By modulating the redox potential and disulfide bond isomerase concentrations, they achieved greater than 95% solubility for a protein containing multiple disulfide bonds. This is in contrast to in vivo studies where correctly folding such complex proteins is very difficult. Indeed, cell-free production of many active disulfide-bonded proteins and disulfide-bonded macromolecular complexes has been achieved, including E. coli alkaline phosphatase, human granulocyte-macrophage colony-stimulating factor, Candida antarctica lipase B, human lysozyme, Gaussia princeps luciferase, and the Qβ virus-like particle which contains up to 180 disulfide bonds (Bundy and Swartz, 2011; Ezure et al., 2007; Park et al., 2009a; Zawada et al., 2011). Although a subtle point, the less-crowded environment of the cell-free reaction (relative to in vivo concentrations) helps provide for improved protein folding.

The open nature of cell-free translation also enables the use of several synthetic approaches to increase solubility of highly insoluble proteins such as membrane proteins (Betton and Miot, 2008). In many instances, detergents have been used to alleviate protein solubility issues (Gilbert G, 2007). In one example, detergents and chaperone proteins were successfully used in the cell-free system to produce an active form of an insoluble cancer-linked protein that had never been produced in vivo (Pedersen et al., 2011). More recently, others have focused on the use of nondetergent surfactants, amphipols, and fluorinated surfactants to increase solubility without

damaging protein structural integrity (Park et al., 2011b). Other recent breakthroughs have included insertion of insoluble proteins into synthetic liposomes with verified postinsertion functionality (Um et al., 2006).

Recently, advances in protein-producing scaffold technology have produced synthetic hydrogels capable of cell-free production yields approaching those of any solution-based cell-free systems (Park et al., 2009b; Park et al., 2009c; Um et al., 2006). Hydrogels are scaffolds made up of linked networks of strongly hydrophilic polymers and mimic hydrophilic physiological environments (Park et al., 2009b). These hydrogels have been reported to be compatible with various cell-free systems, and capable of generating protein yields up to 5 mg/ml (Park et al., 2009c).

2.6 Applications

Improvements in productivity, scale, and complexity of recombinant protein synthesized have rapidly expanded the utility, and now industrialization, of CFPS systems. In this section, we highlight several emerging applications, focusing on protein microarrays, protein evolution, and synthetic proteins.

Currently, the synthesis of proteins for functional analysis, structural genomics projects, or the identification of novel characteristics is a multi-step task. These tasks involve gene/vector construction, protein expression, and functional analysis of the expressed protein. Unfortunately, this process is time-consuming and costly (both in terms of funds and human capital), since it is primarily a serial process where only a single or a few proteins are produced simultaneously. High-throughput production of proteins using CFPS systems is now addressing this challenge. Valuable for studying the synthesis of many proteins simultaneously, protein microarrays are being developed at a rapid pace for improved large- scale protein expression and purification

(Chandra and Srivastava, 2010). The basic principle of protein microarrays is that DNA encoding a protein target of interest is printed onto a glass slide in a physically isolated location, then CFPS synthesizes the target of interest (Ramachandran et al., 2004). To improve protein isolation and stability, proteins are commonly engineered to include epitope tags (e.g. C-terminal glutathione S-transferase tags) which bind the protein products to antibodies immobilized on the slides(Ramachandran et al., 2004). In one exemplary report, parallel large-scale expression of more than 13,000 human genes using a wheat germ extract has been achieved (Goshima et al., 2008). Because the cell-free approach obviates the need to synthesize, purify, and immobilize proteins separately, it allows for proteins with novel characteristics to be quickly generated and analyzed. Further efforts to integrate gene synthesis on chips for protein analysis promise even greater capability.

CFPS offers a rich and versatile platform for protein engineering. Specifically, the cell-free system enables direct control of the environment to efficiently drive protein evolution to contain the desired traits (Zahnd et al., 2007). Two major protein evolution strategies are in vitro compartmentalization and ribosome display. In vitro compartmentalization (IVC) involves the generation of an emulsion to create cell-like compartments (Zhu and Power, 2008). Ideally, each compartment contains a single gene and all reaction components necessary for protein transcription/translation. These separate compartments trap all gene products within an individual compartment, preventing cross-contamination. The reaction components may either be present all at once or added in several phases (Sepp and Choo, 2005). The formation of compartments down to the femtoliter scale has made IVC by emulsion particularly attractive for high-throughput protein evolution (Figure 2-2) (Zhu and Power, 2008). In contrast, the low-tech physical method of IVC by microtiter plate has high reagent costs and the screening of one

million genes could cost over $2.4 million (Fallah-Araghi et al., 2012). The implementation of new ultrahigh-throughput microfluidic systems have dramatically reduced these costs (Agresti et al., 2010). In one such system, the use of picoliter- sized droplets, low polydispersity emulsions, and effective fluorescence sorting allowed the screening of one million genes in less than an hour at a cost of less than $31 (Fallah-Araghi et al., 2012).

As a complement to IVC, ribosome display provides a simple mechanism for evolution of proteins that will bind to a specific ligand (Wada et al., 2008; Yan and Xu, 2006). The ribosome display process begins with a DNA sequence or library of sequences containing a spacer sequence lacking a stop codon. Due to the spacer sequence, the ribosome remains bound to the mRNA_protein complex post-translation and the nascent polypeptide is able to successfully fold (Zahnd et al., 2007). The mRNA_ protein_ribosome complex can then be exposed to a surface-immobilized ligand for binding. Weak-binding complexes are washed away, leaving only high-affinity complexes. The mRNA of the highest affinity complexes are recovered, reverse transcribed, PCR amplified, and then collected for future selection rounds (Yan and Xu, 2006). In one example, ribosome display enabled the rapid selection of a designed ankyrin repeat protein which binds the cancer-relevant epidermal growth factor 2 (Her2) at high selectivity and nanomolar affinity (Zahnd et al., 2006).

Beyond protein microarrays and protein evolution, the synthesis of synthetic proteins is another frontier application of CFPS. While Hecht and coworkers have elegantly shown the ability to synthesize de novo proteins with unique functionality from combinatorial libraries, we focus our discussion here on efforts to expand the chemistry of life by the introduction of unnatural amino acids (Hecht et al., 2004). Efforts to use CFPS for unnatural amino acid incorporation are beginning to grow. This is because of recent advances enabling cost- effective,

high-level CFPS systems, and advantages over in vivo approaches. Namely, there are no transport issues for unnatural amino acids, and there is greater flexibility for reprogramming the translation system (Ohta et al., 2008).

The incorporation of unnatural amino acids to create novel proteins has been performed by globally replacing a natural amino acid with an unnatural analogue, or by site-specifically incorporating the unnatural amino acid while maintaining the natural amino acid cannon. The global replacement method can be performed in the cell-free system by simply not adding an amino acid such as methionine, and adding an unnatural amino analogue in its place. In a recent report, methionine was globally replaced with azidohomoalanine to enable the efficient attachment of proteins to virus-like particles for the development of a B-cell lymphoma vaccine (Patel and Swartz, 2011). While simple in implementation, the global replacement method is more likely to adversely affect protein function due to the loss of methionine from the amino acid canon (Wang et al., 2006).

A number of site-specific incorporation methods have been developed. However, one of the highest yielding and transferable methods involves utilizing an orthogonal tRNA/tRNA synthetase pair specific to the amber stop codon, as developed by Schultz and coworkers (Xie and Schultz, 2006).

The expression or addition of the tRNA/tRNA synthetase pair is relatively straightforward in both in vivo and cell-free systems. However, due to the insoluble nature of some unnatural amino acids and transport limitations of the unnatural amino acid into the cell, the efficient incorporation of some unnatural amino acids in vivo can be challenging (Bundy and Swartz, 2010). Because of the direct access provided by cell-free systems, these limitations can be overcome. By optimizing the tRNA/tRNA synthetase pair and using a continuous exchange cell-

free system with linear templates, Ozawa et al. recently produced protein yields of over 2 mg/ml for a protein with a single unnatural amino acid mutation (Ozawa et al., 2012). For site-specific incorporation, the presence of release factor 1 (RF1) in cell-free extracts can compete with the exogenous tRNA for the amber stop codon (Johnson et al., 2011). A significant improvement in site-specific multisite incorporation of unnatural amino acid protein yields has been made by producing an RF1 knockout strain of E. coli. With this strain, protein yields reached 57 μg/ml for a protein with one unnatural amino acid site mutation, and 68 μg/ml for a protein with three site mutations (Johnson et al., 2011). Although not attempted, the use of this strain in a cell- free system could further improve the production of proteins containing unnatural amino acids.

Small Molecule Metabolites

Similar to CFPS, cell-free metabolic engineering for overproducing small molecule metabolites occurs via either a bottom-up or top-down approach. Since the original discovery that cell extracts could convert sugar into ethanol, there have been relatively few top-down examples of metabolic engineering (Buchner, 1897). Efforts primarily by the Swartz and Panke laboratories have developed the field; the former for activating energy pathways for fueling cell-free protein synthesis reactions (Swartz, 2012), and the latter for making desired small molecule metabolites. In one example of this approach, the Panke group constructed an enzyme catalytic system by fine-tuning a catalytic pathway with information from real-time analysis of concentrations of the pathway intermediates (Bujara et al., 2010).

In contrast to extract-based systems, the bottom-up approach exploits the organization of synthetic enzymatic pathways from purified components, sometimes to facilitate a process or reaction that does not occur in nature (Khalil and Collins, 2010; Zhang et al., 2007b). In one instance, a 13-step synthetic enzymatic pathway using starch and water produced hydrogen at

yields far higher than the theoretical yields of biological hydrogen fermentations (illustrated in Figure 2-3) (Zhang et al., 2007a).

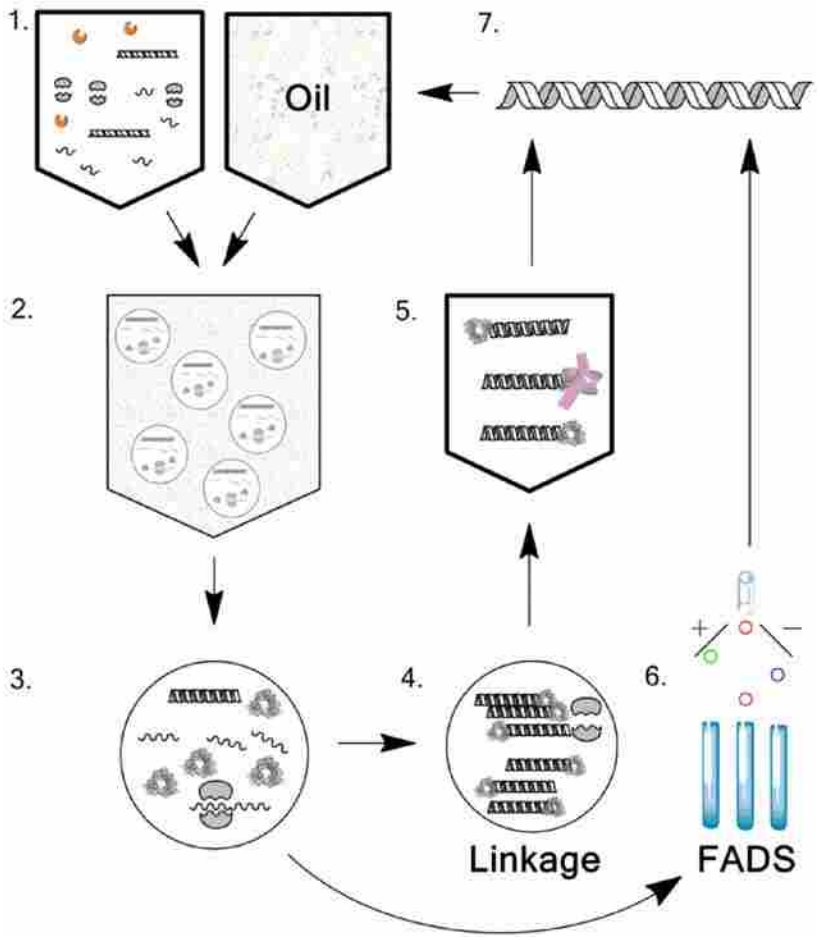


Figure 2-2: In vitro compartmentalization by emulsion for protein evolution. 1-2. Cell-free transcription/translation machinery and mineral oil form water in oil emulsions. 3. In each emulsion, a single gene is transcribed and translated. 4. Protein-gene linkage may be performed prior to selection. 5. Proteins are selected based on binding, catalytic, or regulatory capability. 6. Alternately, proteins may be selected through a fluorescence-assisted droplet sorting system (FADS). 7. The genes for selected proteins undergo a new cycle of IVC. (Ranjil et al., 2013).

Indeed, because carbon flux is not directed towards cell growth, cell-free systems can achieve higher theoretical yields than natural biological processes found in living organisms (Swartz, 2012). Unfortunately, the high cost of purifying stable, standalone enzymes, and

cofactor regeneration costs, currently limit synthetic enzymatic pathways to laboratory scale research.

Cost Reduction

Cell-free biology offers remarkable opportunities for engineering biology. However, until commercial products (e.g. proteins and metabolites) are made using cell-free systems, these systems will mainly serve as test-beds for understanding biology and accelerating the design of synthetic programs. To enable commercial applications, costs must continue to be reduced.

Cells have evolved many pathways to reliably produce energy from inexpensive raw materials. In contrast, more expensive energy sources are typically used in cell-free systems. An overarching goal for cell-free biology is to develop strategies for using the same energy substrate molecules and waste feedstreams that are used to fuel in vivo synthetic biology efforts. Bottom-up approaches will benefit from more efficient methods for rapid and cost-effective protein production and purification, while more efficient and cost- effective extract preparation methods are desired for top-down cell-free systems. With the E. coli-based cell-free protein synthesis system, engineering the extract preparation method decreased the extract preparation time and reagent cost by over 50%, and similar advances are expected to improve other top-down cell-free systems (Kim et al., 2006c; Liu et al., 2005; Shrestha et al., 2014). Further advances will likely couple high-throughput screens to characterize and assess the functions of proteins found in the extract. In one illustrative example, Swartz and colleagues recently surveyed endogenous proteins in E. coli for their influence on CFPS. Using this information, they were able to improve CFPS yields up to 84 g/L in an 8-hour batch reaction (Woodrow et al., 2006).

Scale

With the exquisite control the open cell-free system enables, researchers have

demonstrated the scalability of the cell extract-based cell-free protein synthesis reaction from the microliter level to the 100-liter scale (Zawada et al., 2011). In addition to analogous product protein yields, the production rates at the μL, mL, and L scale were virtually identical (Zawada et al., 2011). Although scalability over six orders of magnitude to the 100 L scale has been demonstrated, increasing to the 1000 L or even 10,000 L scale would enhance the attractiveness of cell-free biology for commercial applications. Moreover, the development of a scalable eukaryotic system is needed.

Development of Complex Biocatalytic Networks

The cell-free system provides an environment where the expression rate of multiple products can be controlled in parallel to assess the impact of coproduction on an individual product's activity. This is particularly important when assembling complex macromolecular assemblies such as the ribosome or virus-like particles. As recently demonstrated, small changes in expression levels of one protein of multiprotein macromolecular complex can significantly impact the efficiency and activity of the assembled macromolecular complex (Smith et al., 2011). In addition, researchers have noted the engineering advantages of employing in vitro enzyme-catalyzed biochemical reaction cascades to produce the desired natural product (Santacoloma et al., 2010). The easily controlled and engineered open cell-free environment will be exploited for optimizing multienzyme synthesis and activity for next-generation biocatalysts. Efforts such as those by Forster and colleagues could provide directions (Du et al., 2009; Du et al., 2012).

Purification and Localization of Multienzyme Ensembles

To produce a desired product from an inexpensive feedstock, multiple biocatalytic steps are often necessary. A key challenge, therefore, for bottom-up cell-free systems is cost-effective

purification of multienzyme ensembles. Offering one possible solution, a recent report from Wang et al. leverages advanced genome engineering for simultaneous modification of catalysts to be used in copurification strategies (Wang et al., 2012). This type of strategy, once further developed, could lead to commercially relevant in vitro catalytic systems. Beyond strategies for simultaneously modifying and copurifying large protein complexes and pathways, localization may also be important. Nature has evolved highly efficient mechanisms to facilitate the transfer

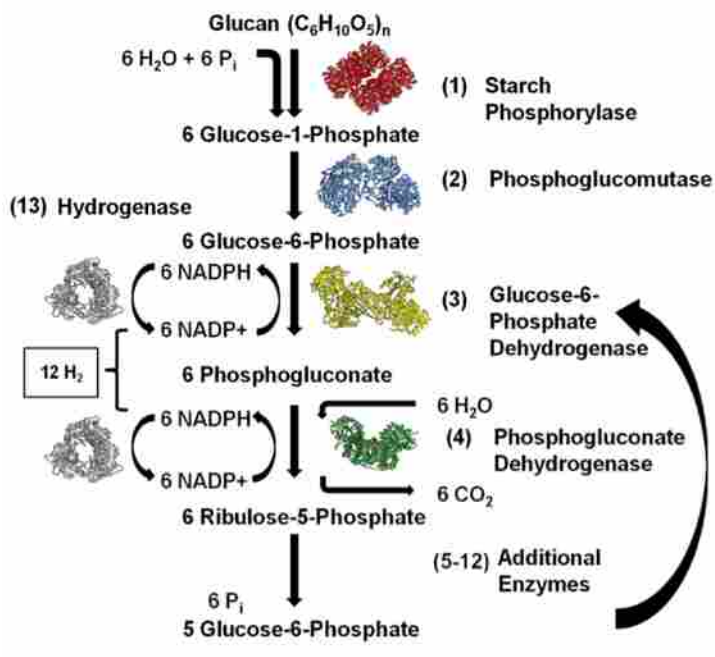


Figure 2-3: A cell-free synthetic enzymatic pathway for converting starch and water into hydrogen and carbon dioxide: This artificial pathway has been reported to produce hydrogen at yields greater than the theoretical limits for industrial anaerobic fermentations (12 H_2 per glucose compared to4 H_2 per glucose)(Zhang et al., 2007a). Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081). (Ranjil et al., 2013).

of reactants through complex enzymatic pathways. For the production of secondary metabolites, for example, type I polyketide synthetases actively transfer the reactant through a number of catalytic domains that are locally constrained as part of the same protein. Biomimetic engineering, the ability to control the proximity of multiple enzymes, could facilitate the rapid

transfer of reactants through the correct sequence of biocatalytic events. Innovations in the rapidly growing enzyme immobilization field could help facilitate the development of such biomimetic multienzyme ensembles(Sheldon, 2007).

2.7 Conclusion

Cell-free synthetic biology is a powerful tool for understanding and expanding the basic biology of living systems. Recent advances in nucleic acid circuitry, protein synthesis, and small molecule production, amongst others, have heralded a new age of in vitro bioengineering. The general simplicity and flexibility of in vitro systems serves as an excellent complement to in vivo studies, and enables a more complete understanding of the inner workings of a cell. Cell-free synthetic biology has versatile applications in protein synthesis and evolution, as well as the development of nucleic acid circuits, nanomachines, and therapeutics.

Although cell-free systems have been used for over 50 years, more work is needed before they can be used as commercial platforms for therapeutic and metabolite production. However, recent advances have demonstrated commercial-scale protein synthesis with crude extract cell-free systems. In addition, continued engineering has rapidly driven down the cost and simplified the preparation of many cell-free systems. With the remarkable progress made over the last 10 years, it is but a matter of time before several of the significant challenges facing the field evolve into transformative synthetic biology opportunities.

3. Enhanced Protein Stability Through Minimally Invasive, Direct, Covalent, and Site-specific Immobilization

This chapter is an adaptation from the January 2013 Biotechnology Progress article entitled "Enhanced Protein Stability Through Minimally Invasive, Direct, Covalent, and Site-specific Immobilization." This research project was led by me in conjunction with a fellow graduate student, Mark Smith, with the help of an undergraduate researcher, Christopher Werner.

3.1 Introduction

Proteins represent a $100+ billion USD industry with rapid annual growth (Sarrouh, 2012). The utilization of proteins for bioprocessing, sensing, and medical treatment is widespread over a number of industries including agricultural, textile, pharmaceutical, energy, and chemical processing (Ragauskas et al., 2006; Schoemaker et al., 2003). Proteins have a number of advantages over nonbiological chemicals and chemical processes due to: (1) superior chemo-, regio-, and stereo specificity of enzymes (Bornscheuer, 2003; Grunwald, 2010; Patel, 2001; Schoemaker et al., 2003); (2) green sustainability (renewable, biodegradable, nontoxic, milder reaction conditions, less energy consumption) (Alcalde et al., 2006; Ragauskas et al., 2006; Schmid et al., 2001; Schoemaker et al., 2003); and (3) optimization through protein engineering (Vick and Schmidt‐Dannert, 2011). However, difficulties in recovery, reuse, and long-term stability limit the far-reaching industrial potential of proteins (Sheldon, 2007). Protein immobilization is a potential solution as it simplifies protein recovery and reuse, and reportedly increases protein stability in many cases (Adamczak and Krishna, 2004; Alcalde et al., 2006; Bornscheuer, 2003). This work is focused on improving protein immobilization and we introduce the Protein Residue-Explicit Covalent Immobilization for Stability Enhancement or PRECISE system that enables the targeted immobilization of proteins to enhance protein stability and durability.

The most robust immobilization techniques for protein-surface bioconjugation are covalent (Dyal et al., 2003; Wang et al., 2009a). However, precisely controlling which location on the protein covalently binds to a surface is complicated by the naturally limited pool of only 20 amino acid building blocks. Covalently targeting a given amino acid which appears in multiple locations in a protein commonly results in undesirable attachment orientations and loss of activity and stability (Ladavière et al., 1998; Rusmini et al., 2007; Stephanopoulos and Francis, 2011).

A number of site-specific yet noncovalent approaches have demonstrated superior activity using recognition pairs such as 6His/Ni,20 biotin/streptavidin (Yu et al., 2012), more generally oligonucleotide/protein (Niemeyer, 2002). However, long-term stability and reusability are compromised due to protein leaching that is further exacerbated under industrially relevant conditions (Rusmini et al., 2007). In addition, such systems commonly require the fusion of large tags which can negatively affect protein structure and function especially if inserted at locations other than the terminus (Carson et al., 2007; Rusmini et al., 2007).

Increasingly, research efforts have focused on developing both a covalent and site-specific immobilization technique. Specific targeting of the C and N termini for conjugation to peptide tags has been successfully reported with tags such as transglutaminase (Tanaka et al., 2007) and phosphatase (Gauchet et al., 2006), yet remains insufficient in cases where the termini are critical to protein folding and/or function. To target residues within the protein, complete elimination of all similar residues and chemical moieties except at the desired conjugation site can enable site-specific covalent linkage (Ladavière et al., 1998). However, mutagenesis of this magnitude is typically destabilizing. Alternatively, unnatural amino acids (uAAs) provide unique functional groups which can react with a number of bioorthogonal and highly efficient reaction chemistries

such as click chemistry, the Staudinger reduction, and Diels–Alder reaction-like cycloadditions (Boyce and Bertozzi, 2011; Rusmini et al., 2007; Stephanopoulos and Francis, 2011). Global replacement of a natural amino acid with an uAA analogue achieved by omission of the natural amino acid during protein synthesis is commonly performed, although, site-specific immobilization is still complicated by the incorporation of the unnatural amino acid at many locations (Patel and Swartz, 2011; Strable et al., 2008).

The PRECISE system overcomes the reported issues of nonspecific and noncovalent immobilization by building upon a developed technology that site-specifically incorporates a single uAA at a single explicitly predetermined residue (Patel and Swartz, 2011; Wang et al., 2001a; Young et al., 2010). This technology was initially developed as an in vivo Escherichia coli-based system that uses orthogonal tRNA and aminoacyl-tRNA synthetase from Methanocaldococcus jannaschii to incorporate the uAA only in the location encoded by the amber stop codon (UAG) on the mRNA (Wang et al., 2001a; Xie and Schultz, 2006). Using this method, over 70 uAA have been incorporated with generally high selectivity (Liu and Schultz, 2010; Wang et al., 2002). Two particularly attractive unnatural amino acids are p-propargyloxyphenylalanine (pPa) and p-azidophenylalanine (pAz) which can be used with the highly selective, efficient, and biocompatible copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition click reaction. Because of the photoinstability of pAz, this work focuses on the incorporation of pPa to enable direct click chemistry to the surface.

Using the in vivo site-specific insertion technique, Kim et al. targeted a predetermined residue location in the protein DrrA and clicked an alkyne-biotin with site-specifically incorporated pAz (Seo et al., 2011). The biotin-linked protein was then surface-immobilized by the noncovalent neutravidin-biotin interaction. Compared to randomly oriented immobilized

DrrA, the orientation-specifically immobilized DrrA exhibited higher activity of DrrA(Seo et al., 2011). Although Kim et al. demonstrated the capability of residue-explicit targeting, the noncovalent link limits the immobilization's robustness.

The PRECISE system uses site-specific uAA and direct covalent immobilization, eliminating the need for a noncovalent linker. In addition, the photostable pPa is incorporated using an E. coli-based cell-free protein synthesis (CFPS) approach that eliminates membrane-transport limitations and has resulted in up to a 27-fold increase in protein production yields for proteins containing pPa (Bundy and Swartz, 2010). Other advantages of CFPS include direct access and optimization of the synthesis environment, simplified purification, and high-throughput automation potential (Bundy and Swartz, 2011; Kim et al., 2011; Shrestha et al., 2012).

The site-specific covalent surface immobilization of proteins presented herein provides a unique balance of stable protein binding and orientation control without the need of noncovalent linkers. While the technology for the site-specific incorporation of pPa using cell-free protein synthesis has been previously reported (Bundy and Swartz, 2010), here we report the application of this technology for site-specific immobilization of proteins onto nonprotein surfaces such as superparamagnetic beads. Our results also indicate extended longevity and stability of active bound proteins under biologically unfavorable conditions with potential implications for industrial biocatalysis, protein microarrays technologies and other protein based diagnostic applications.

3.2 Materials and Methods

3.2.1 Extract preparation

E. coli extract was prepared using a BL21 Star™ (DE3) E. coli strain purchased from

Invitrogen (Carlsbad, CA) harboring the pEVOL-pPrF plasmid, a kind gift from Peter Schulz, (Scripps Research Institute). The resulting E. coli strain produces the Methanocaldococcus jannaschii aminoacyl-tRNA synthetase/tRNA pair capable of recognizing and inserting pPa at the amber stop codon AUG.30 Cell extract was prepared as previously reported (Bundy and Swartz, 2010; Bundy and Swartz, 2011) with the following modifications. The strain was fermented in 1 L batches of 2xYT media buffered with 100 mM MOPS at 37°C and shaken in a 2.5 L Tunair baffled shake flask (IBI Scientific, Peosta, IA) at 280 rpm. Cells were lysed through triple pass homogenization using an Avestin Emulsiflex B-15 cell disruptor (Ottawa, Canada) at 21,000 psi. Subsequently, the remaining cell extract was flash-frozen and stored at −80°C until used.

3.2.2 Cell-free protein synthesis

Protein synthesis of T216pPa sfGFP (pPaGFP) was carried out in vitro using the PANOx-SP system as previously described with the following minor modifications (Bundy and Swartz, 2010). Plasmids used were either pY71-sfGFP-Strep or pY71-T216Amb-sfGFP-Strep. Unless specified, all reactions contained 2 mM pPa. BL21 Star™ (DE3) E. coli extract was added at 25% v/v, without additional T7 RNA polymerase. Reactions contained 7 μg mL−1 Methanocaldococcus jannaschii aminoacyl-tRNA synthetase affinity purified according to a previously reported procedure(Goerke and Swartz, 2009). Reactions were performed at 30°C for 8 h or overnight.

3.2.3 Protein yield assays

Total and soluble protein yield calculations were performed using liquid scintillation counting as discussed previously (Bundy et al., 2008; Smith et al., 2011). An analysis of full-length protein yields was performed by first running sodium dodecylsulfate polyacrylamide gel

electrophoresis on the supernatant of the CFPS reaction mixtures after centrifugation at 13,000g for 15 min, and on strep-column purified protein as per the manufacturer's specifications (Life Technologies, Carlsbad, CA). Autoradiograms were prepared on Kodak MR Autoradiography Films (Rochester, NY) and densitometry calculations were performed using ImageJ software.43

3.2.4 Fluorescence activity assay

Fluorescence assays were performed using a Synergy MX (Biotek Instruments, Winooski, VT) at 485/510 nm excitation/emission and sensitivity of 100 in 60 or 125 μL of PBS buffer per well of a black-bottom 96-well plate.

3.2.5 Dynabead preparation

The superparamagnetic M-270 Dynabeads decorated with terminal amines were reacted with azide-containing ligands, using a procedure adapted from Punna et al (Punna et al., 2005). First, 4-nitrophenyl 5-azidopentanoate was synthesized through the addition of 5-azidopentanoic acid (3.5 mmol) and p-nitrophenol (3.7 mmol) to a solution with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (3.7 mmol) in CH2Cl2 at room temperature overnight. The 4-nitrophenyl 5-azidopentanoate was then concentrated and purified using silica gel column chromatography.

After purification, the 4-nitrophenyl 5-azidopentanoate was dissolved in DMF and added to a tube containing 2 mL of Dynabeads (2 × 109 beads per mL) in 5% MES buffer and stirred gently at room temperature for 2 days. Measurement of the released p-nitrophenol absorbance at

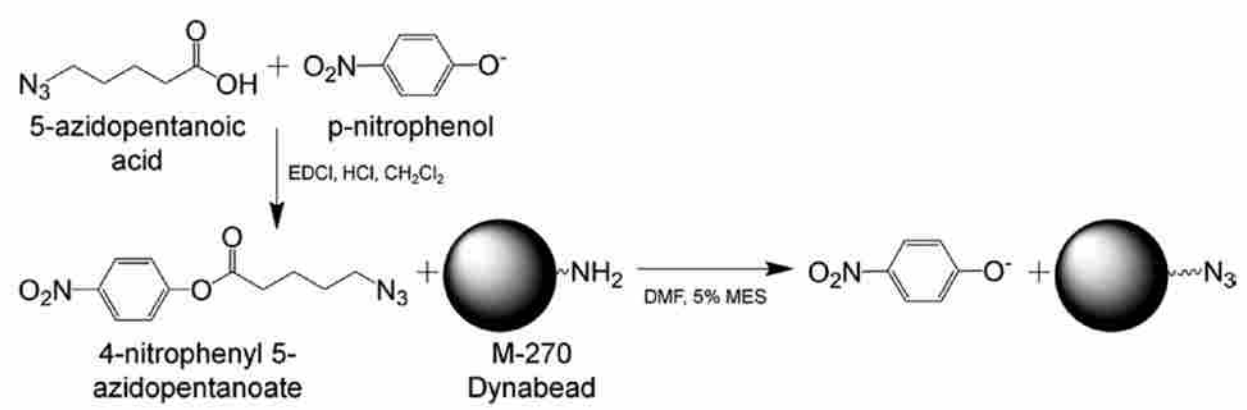


Figure 3-1: Preparation of Azide-functional Surface (Smith et al., 2013).

412 nm was then performed and compared to a p-nitrophenol standard curve. A negative control reaction containing no beads (no amines) was performed and no change in 412 nm absorbance was observed suggesting no autohydrolysis of the 4-nitrophenyl 5-azidopentanoate. Beads were vigorously washed 10 times with PBS buffer to remove excess and non-specifically adsorbed azide ligand.

3.2.6 Click reaction (copper-catalyzed azide-alkyne cycloaddition)

The aqueous click reaction conditions were performed with either 1 mM CuSO4 and 5 mM sodium ascorbate or 1 mM tetrakis(acetonitrile)copper(I) hexafluorophosphate (Cu(I)Tet) for CuSO4 or Cu(I)Tet catalyzed reactions, respectively. Click reactions with Alexa Fluor® 555 alkyne (AF555) (Invitrogen, Carlsbad, CA) were performed exclusively with Cu(I)Tet with 0.2–20 nmol of AF555 in 30–50 µL reaction volumes. The reaction volume for click reactions with pPaGFP was 100 µL with 0.5 nmol pPaGFP and 5 nmol azide-functional groups in the reaction. Reagents were prepared aerobically in microcentrifuge tubes with all components save the catalyst and sodium ascorbate. Tubes were then transferred to an anaerobic glove box atmosphere containing <0.0 ppm oxygen and allowed to degas for a minimum of 45 min. After degassing, catalyst and, if required, sodium ascorbate was added. The closed tubes were then

rotated end-over-end for 8–14 h in darkness. Reactions were terminated by removal of the tubes from the anaerobic glove box and exposure to atmospheric oxygen.

3.2.7 Purification of click-immobilized pPaGFP

To purify the superparamagnetic beads from the click reaction solution, a DynaMag Spin Magnet (Invitrogen, Carlsbad, CA) was used as follows. The microcentrifuge tubes containing the click reaction were placed on the DynaMag Spin Magnet for 8 min, after which the supernatant was removed. The tubes were then removed from the magnet, 100 μL PBS-Tween buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 0.05% v/v Tween20) was added to the samples, the samples were thoroughly vortexed, and the new suspensions were incubated for 8 min at room temperature. Samples were returned to the DynaMag Spin Magnet and the cycle was repeated two times. After the final removal of the supernatant, the sample was resuspended in 100 μL PBS.

3.2.8 Freeze thaw

pPaGFP was immobilized on azide-functional Dynabeads using both CuSO4 and Cu(I)Tet and purified as described above. In a single black 96-well plate (Plate A), both pPaGFP immobilized on beads and free pPaGFP was aliquoted into different wells to a final volume of 60 μL in PBS buffer. In a separate black 96-well plate (Plate B), free pPaGFP was aliquoted into different wells to a final volume of 60 μL in PBS buffer. Plate A was subjected to four freeze-thaw cycles by incubating it at −80°C for 20 min and then incubating it at room temperature for 20 min. Between each freeze-thaw cycle fluorescence measurements of the pPaGFP were taken with the Synergy MX microplate reader as described above. Concurrently, Plate B was incubated at 4°C and fluorescence measurements of the pPaGFP were also taken every 40 min.

3.2.9 Urea/heat incubation

pPaGFP were immobilized on azide-functional Dynabeads using both CuSO4 and Cu(I)Tet and purified as described above. In a single black 96-well plate (Plate C), both pPaGFP immobilized on beads and free pPaGFP were aliquoted into different wells to a final volume of 50 μL in PBS buffer. To all wells containing pPaGFP immobilized on beads and to half of the wells containing free pPaGFP was added 75 μL of 10 M urea, bringing the final urea concentration to 6 M. To the remaining wells of free pPaGFP was added 75 μL of deionized sterile water in place of urea. In a separate black 96-well plate (Plate D), free pPaGFP was aliquoted into different wells to a final volume of 50 μL with PBS buffer following which 75 μL of deionized sterile H2O was added.

Immediately after adding urea or water, Plate C was placed in the Synergy MS microplate reader with the sample chamber temperature maintained at 50°C and assayed for fluorescence activity every minute for 30 min. The plate was removed from the plate reader and cooled to 4°C and assayed for fluorescence activity after 1.5 and 12 h. Place D was treated in a similar manner with the exception of the microplate reader's sample chamber temperature being maintained at room temperature.

3.3 Results and Discussion

The protein residue-explicit covalent immobilization for stability enhancement or PRECISE system introduced herein allowed for retention of protein activity and increased the stability and durability of the proteins. Azide-functional superparamagnetic beads were prepared as the attachment surface and tested using small alkyne-functional fluorophores. Then, GFP containing an unnatural amino acid (uAA) was synthesized, purified, and successfully immobilized to the beads with the PRECISE system as illustrated in Figure 3-1. Immobilized

GFP maintained a significant fluorescence activity and exhibited increased stability against freeze-thaw cycles, urea and elevated temperature compared to unbound GFP, as will be described in detail below.

3.3.1 Preparation of Azide-functional Surface

Superparamagnetic beads were selected as the immobilization surface for simplicity in purification, recovery, and reuse. Furthermore, they provide a macro, inorganic surface for conjugation. Amine-decorated superparamagnetic beads (Dynabeads® M-270, Invitrogen) were modified with terminal azide groups as shown in Figure 3-2. To make the beads viable reagents in the click reaction, the amine-coated beads were reacted with 4-nitrophenyl 5-azidopentanoate and the release of p-nitrophenol was measured by absorbance to determine extent of reaction, which resulted in azide-functionalization of 95% of the amine groups available as per the manufacturer's specifications (Invitrogen).

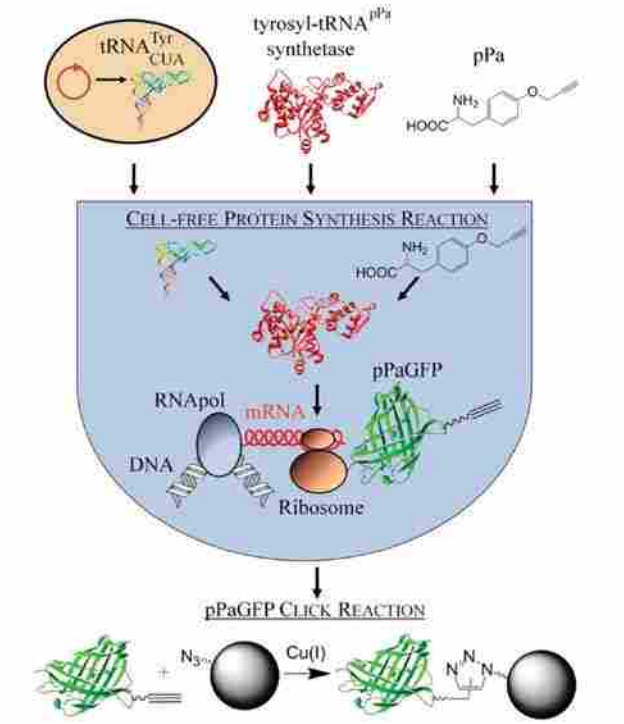


Figure 3-2: The PRECISE System (Smith et al., 2013).

3.3.2 Cell-free Protein Synthesis

Superfolder green fluorescent protein (sfGFP) was rationally engineered to incorporate an uAA at T216 and to contain a C-terminal purification tag (Bundy and Swartz, 2010). The site T216 was chosen for substitution by identifying a surface-accessible protruding loop near an end of the cylindrical protein's crystal structure. The C-terminal Strep-tag provided means of purifying full-length protein from truncated product, which occurs when the uAA is not incorporated and results in early termination of the protein (Figure 3-2).

By carefully choosing the location of mutagenesis and avoiding alterations to the main structural or active portions of the protein, the potential for improper folding or activity loss is minimized. Indeed, the activity of purified uAA-containing sfGFP did not drop compared to the activity of unmodified sfGFP (Bundy and Swartz, 2010).

The PRECISE system utilizes the open access afforded by CFPS to optimize concentrations of the Methanocaldococcus jannaschii aminoacyl-tRNA synthetase, which works together with its tRNA pair to incorporate the uAA pPa at the Amber stop codon. As displayed in Figure 3-3 pPa was successfully incorporated ~40% of the time when pPa was present in the CFPS reaction. When pPa was absent, the protein was truncated at the amber stop codon with no detectable incorporation of pPa (Figure 3-3, Lane 3), which is consistent with previously reported works (Hohsaka et al., 2001; Saraogi et al., 2011).

The CFPS production approach has specific advantages over in vivo protein production such energy focused toward expression of the product protein(s), simplified product purification, and elimination of transport-limiting cell-walls (Bundy and Swartz, 2011). In addition, CFPS enables direct access to the open synthesis environment and enables facile optimization and

maximization in the concentration of synthetase, uAAs, and other cofactors essential for protein synthesis.

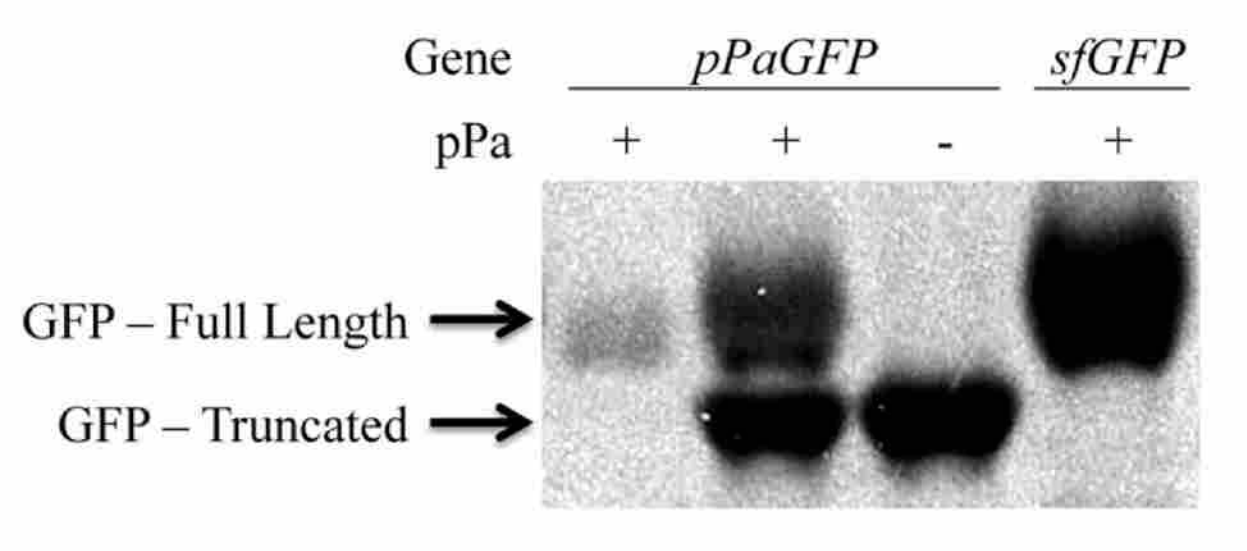


Figure 3-1: SDS-PAGE and Autoradiogram of pPaGFP. The lanes are laid out from left to right as follows: (1) pPaGFP post-Strep-tag purification; (2) synthesized pPaGFP prior to purification; (3) pPaGFP synthesized without the presence of pPa; and (4) unmodified sfGFP synthesized under otherwise identical conditions to other products. (Smith et al., 2013).

3.3.3 Click Immobilization

Preliminary click immobilizations to the beads exploring click conjugation efficiency were performed using a small fluorescent ligand, Alexa Fluor® 555 alkyne (AF555). Click conjugation was successful under multiple reagent concentrations as seen in Figure 3-4. Reactions containing 1 nmol or more AF555 resulted in the highest amount of immobilization. At 5 and 20 nmol AF555, the resulting attachment was not statistically higher than at 1 nmol (P value = 0.78). This apparent maximum results in an average of 12 million AF555 per bead. The number of AF555 attached and the percentage attached in Figure 2 are conservatively reported and do not account for signal lost due to light diffraction and shadowing caused by the beads. The number of AF555 attached and the percentage attached are conservatively reported above

and do not account for signal lost due to light diffraction and shadowing caused by the beads.

Reaction conditions for pPaGFP immobilization were selected based on AF555 results. pPaGFP were immobilized onto the azide-functional magnetic beads by anaerobic incubation using reduced copper from either CuSO4 or tetrakis (acetonitrile)copper(I) hexafluorophosphate (Cu(I)Tet) as a catalyst. The results are reported in Table 3-4. The Cu(I)Tet-catalyzed reactions resulted in a higher click attachment percentage at 11% (9.6 million pPaGFP per bead), compared to the CuSO4− catalyzed reactions at 4.5% (3.9 million pPaGFP per bead). The two catalyst systems provide the essential catalyst (Cu(I)) with differing mechanisms. Cu(I)Tet provides Cu(I) directly while the CuSO4 system relies on the redox potential of sodium ascorbate to reduce Cu(II) to Cu(I). The divergence in catalyst-recovery mechanisms is a likely cause for the mild divergence in overall reaction efficiency. While high reaction efficiencies are reported in literature for similar click reactions using protein reagents (Hong et al., 2009; Li et al., 2012; Patel and Swartz, 2011), the regent concentrations used in this work are at least an order of magnitude lower. Excellent work by M.G. Finn's lab (Scripps Research Institute) has demonstrated that the optimal copper catalyst concentration is dependent upon the reagent concentration (Hong et al., 2009; Presolski et al., 2010). In addition, Finn and others have reported a variety of ligands which improve the kinetics and reaction efficiency and the optimal concentration of such ligands can depend on the reagent types and concentrations (Hong et al., 2009; Presolski et al., 2010). Further work to optimize the click reaction at the lower concentrations used in this work, could increase the reaction efficiency. However, we do observed significant pPaGFP attachment with the reported reaction conditions.

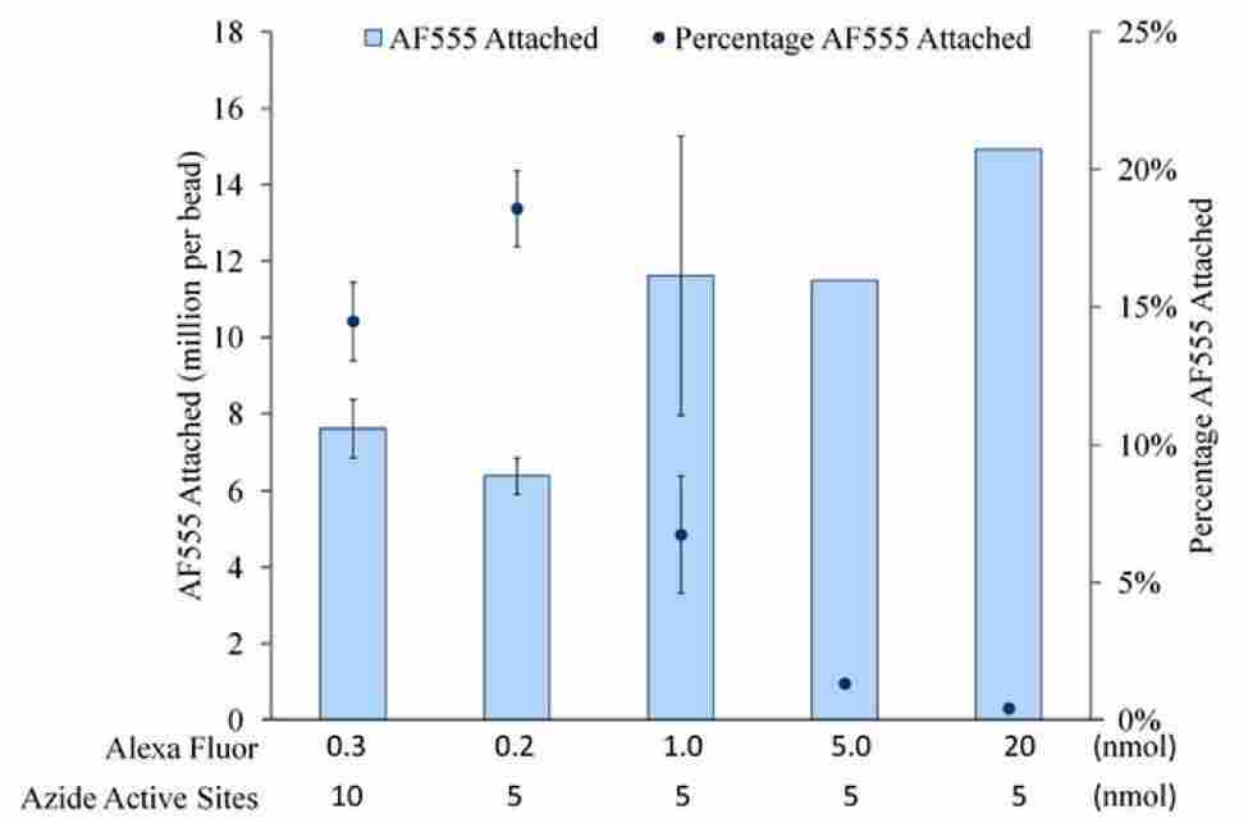


Figure 3-2: Alexa Fluor 555 and Bead Click-Conjugation Profile. The number of AF555 attached and the percentage attached are conservatively reported above and do not account for signal lost due to light diffraction and shadowing caused by the beads. ‘‘Percentage AF555 Attached’’ refers to the percentage of AF555 attached compared to the amount input into the reaction solution. No background attachment of AF555 to beads was evident under any of the conditions considered. Error bars = standard deviation (n=2) or none (n=1). (Smith et al., 2013).

Adsorbed proteins were incubated with azide-decorated beads in the absence of copper and then washed with PBS buffer in lieu of PBS-Tween buffer (n = 1). Similar trials washed in PBS-Tween buffer resulted in essentially no detectable adsorption (n = 2). See Supporting Information After washing the beads with PBS-Tween buffer three times, nonspecific adsorption was insignificant. However, washing with PBS only resulted in some nonspecific adsorption (0.5 million pPaGFP per bead as compared to 3.9 and 9.6 million pPaGFP per bead attached via click conjugation).

The cyclo-addition of azides to nonactivated alkynes is Cu(I)-catalyzed to avoid high

pressures and temperatures that would be incompatible with biological system (Prescher and Bertozzi, 2005) However, copper ions can quench GFP fluorescence by direct, nondenaturing interaction with the chromophore (Barondeau et al., 2002; Jung et al., 2005). In addition, it is difficult to accurately assess the specific activity of the immobilized protein due to the complicating factors of bead-induced light diffraction and shadowing, which may cause up to 50% signal reduction. However, based on the very conservative assumption that all active pPaGFP on the bead were observed (i.e., no loss due to the bead-induced light diffraction and shadowing), immobilized pPaGFP retained at least 40% of the activity of the free pPaGFP incubated in the click reaction environment. For clarity, when reporting the stability of immobilized pPaGFP and free pPaGFP under the harsh conditions below, changes in activity per mol pPaGFP are reported to eliminate the impact of bead-induced signal loss factors.

Table 3-1: pPaGFP Attachment and Observed Activity Values

Unbound[a]	$CuSO_4$	Cu(I)Tet	PBS Buffer
Incubated activity [RFU/mol pPaGFP]	37.0 ± 0.01	8.4 ± 3.0	109.7 ± 11.7
Bead-conjugated	$CuSO_4$	Cu(I)Tet	Adsorbed[b]
Percent pPaGFP attached	4.5% ± 1.2%	11.3% ± 3.5%	0.6%
Overall attached [million per bead]	3.9 ± 1.0	9.6 ± 3.0	0.5
Observed activity attached [RFU/mol pPaGFP]	15.6 ± 4.4	4.2 ± 1.9	12.0

For all data n = 2 with ± standard deviation unless specified.
[a]Unbound protein were incubated in the respective reaction solutions and then assayed for activity without beads present.
[b]Adsorbed proteins were incubated with azide-decorated beads in the absence of copper and then washed with PBS buffer in lieu of PBS-Tween buffer (n = 1). Similar trials washed in PBS-Tween buffer resulted in essentially no detectable adsorption (n = 2).

3.3.4 Freeze Thawing

To evaluate if the immobilized pPaGFP exhibited improved stability, free pPaGFP and bead-immobilized pPaGFP were subjected to multiple cycles of freezing and thawing. Changes in pPaGFP activity were normalized per mol pPaGFP and are reported in Figure 3-5. The bead-immobilized pPaGFP activity increased slightly over the four freeze-thaw cycles. In contrast, freeze-thawed free protein lost an average 17% of the initial activity after each cycle, resulting in a 68% loss in activity after four freeze-thaw cycles. Free protein kept at 4°C over the entire experiment also resulted in a drop in activity after four cycles. Free proteins subjected to freeze-thaw or maintained at 4°C in the presence of the copper-catalyst of the click reaction exhibited very similar trends as their copper-free counterparts.

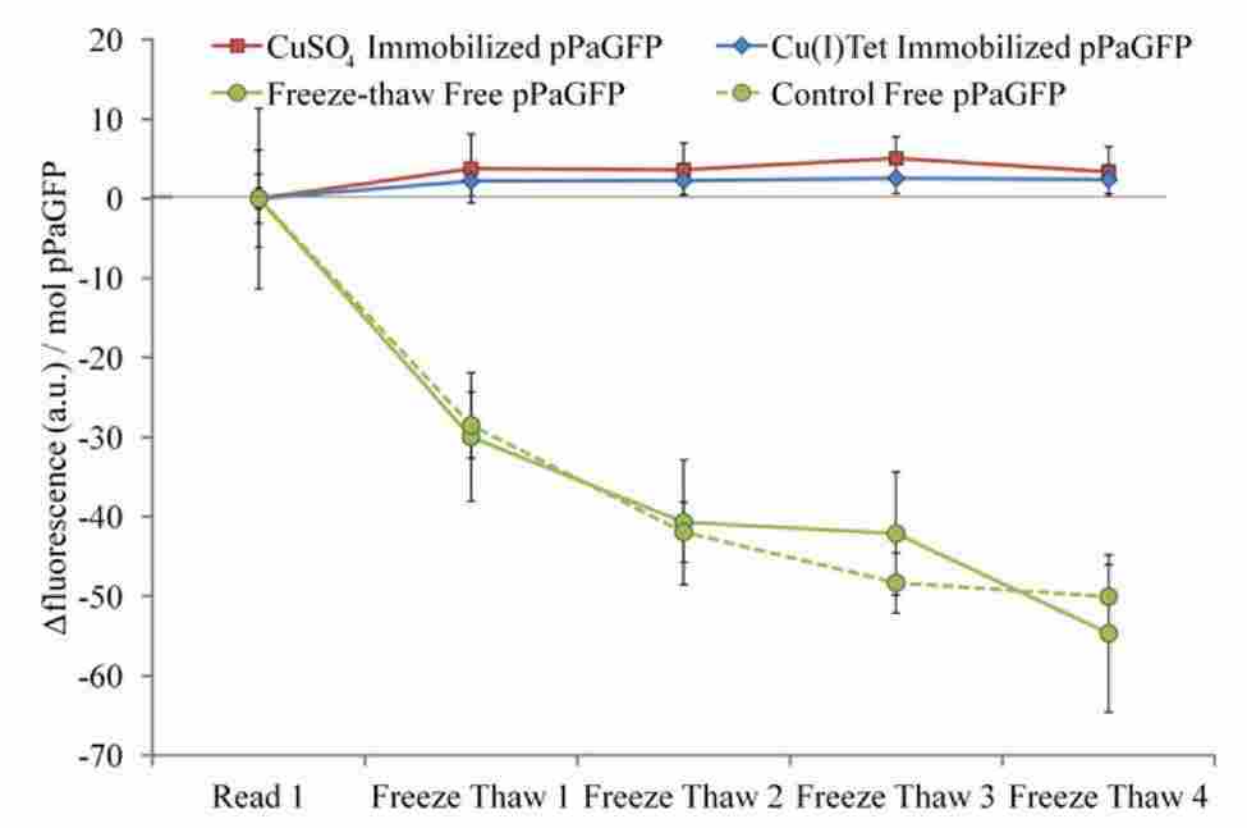


Figure 3-3: Freeze-thaw Effects on Stability. Bead-conjugated pPaGFP were run in parallel through freeze thaw cycles (20 min at −80°C, 20 min at 25°C, assay, repeat) following which the fluorescence activity was assayed. In addition, a control for the behavior of free pPaGFP

consisted of free pPaGFP either freezethawed (solid line, circles) or maintained at 4°C (dashed line, circles). Errors bars = standard error (n = 2 for beads, 26 for freeze-thawed, 50 for control). (Smith et al., 2013).

3.3.5 Urea/Heat incubation

Bead-immobilized pPaGFP and free pPaGFP were incubated under the harsh conditions of urea (6 M) and elevated temperature (50°C). Resulting changes in activity were normalized per mol pPaGFP and are reported in Figure 3-6. Bead-immobilized proteins retained 57% or more of their initial activity throughout the duration of the incubation in urea at 50°C. After the samples were cooled to 4°C, the activity of immobilized pPaGFP recovered 83% or more of the initial value, despite continued incubation in urea. In contrast, free pPaGFP retained <10% of their initial activity during heating and after 12 h of cooling exhibited <3% of the initial activity, regardless of urea's presence or absence.

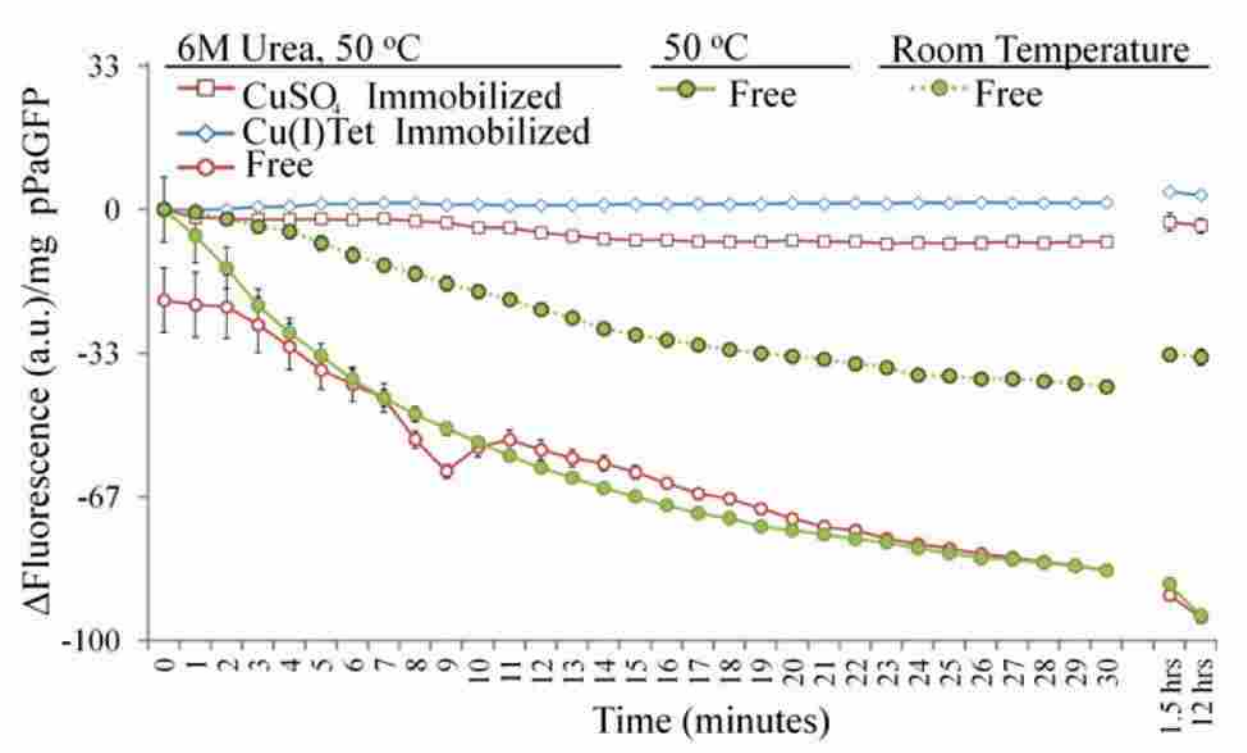


Figure 3-4: Urea and Heat Effects on Stability. Bead-conjugated pPaGFP were run against multiple controls and assayed for fluorescence. The solutions, with the exception of the free

pPaGFP at room temperature, began at room temperature and were incubated at 50°C for the duration of the assay directly after addition of room-temperature urea or water. The addition of urea caused the free pPaGFP to drop in activity prior to any heating (empty circles). The bead-immobilized pPaGFP did not exhibit any immediate loss in activity due to the addition of urea (empty squares/diamonds). Error bars = standard error (n = 2 for bead-conjugated pPaGFP, n = 3 for free pPaGFP, and n = 4 for free room temperature pPaGFP). (Smith et al., 2013).

3.4 Conclusion

We have introduced an example of Protein Residue-Explicit Covalent Immobilization for Stability Enhancement or PRECISE system. Click chemistry teamed with minimally invasive site-specific uAA-incorporation using cell-free protein synthesis allowed for the rationally engineered protein to be directly and site-specifically immobilized onto an inorganic surface. In all cases explored here, the immobilized pPaGFP appeared to exhibit superior stability, durability and retained activity during multiple harsh scenarios as compared to free protein in solution. The ability to site-specifically attach proteins to a surface using bio-orthogonal chemistry at any predetermined site has provided a basis for more stable and longer lasting protein activity. This technology has numerous implications in the industrial, scientific and medical communities and could be applied to enhance industrial enzyme recovery, simplify laboratory assays and machinery, and reduce medical diagnostic costs while increasing consistency, availability and transportability. Furthermore, this work solidifies the foundation for the PRECISE system for highly stable protein immobilization.

4. Enhancing Candida antarctica Lipase B Activity, Stability, and Reusability Through Rationally Directed, Site-specific Immobilization

4.1 Introduction

Chemical catalysis provides one of the foundational blocks upon which the $812 billion American chemical industry is built (based on 2014 American Chemistry Council estimates). Due to the increased demand for environmentally friendly, sustainable, and highly specific chemical manufacturing processes, biocatalysis is rapidly growing as a replacement for traditional catalytic techniques (Clark, 2009). Enzyme catalysis has several advantages that make it particularly suited for chemical manufacturing purposes: biocatalysts are biodegradable, non-toxic, can be performed in aqueous conditions and at ambient temperatures, are reusable/recoverable, do not require toxic reagents, and are highly specific (Alcalde et al., 2006; Sheldon, 2007). Further growth in the biocatalysis industry relies upon solutions to a few enzyme use challenges, primarily, the need to maintain enzyme stability over time and enzyme catalyst recoverability and reusability (Ragauskas et al., 2006). One solution to the difficulties of increasing enzyme stability over time and enzyme recoverability and reusability is the immobilization of enzymes on a surface or carrier.

Enzyme immobilization has been shown in many instances to increase enzyme stability and recoverability/reusability (Sheldon, 2007). Moreover, enzyme immobilization greatly simplifies enzyme recoverability and reuse, greatly reducing the cost of enzyme use over time. The most common enzyme immobilization techniques, utilizing adsorption and ionic immobilization processes, have enabled greater enzyme recoverability and reuse but are limited by the issues of enzyme stability and leaching over time. Covalent enzyme immobilization resolves the issue of enzyme leaching and improves enzyme stability but like both adsorption and ionic immobilization, do not enable immobilization orientation control.

Although the importance of enzyme immobilization orientation control has been suggested by molecular modeling simulations and in a limited fashion by previous enzyme immobilization technologies that limited potential immobilization sites, covalent immobilization orientation control has not been thoroughly studied (Tanaka et al., 2007; Wei and Knotts, 2011a). Previous site-specific immobilization techniques have relied on techniques such as global replacement or N- or C-terminus immobilization that do not permit adequate testing of the impact of immobilization orientation on enzyme activity (Gauchet et al., 2006; Tanaka et al., 2007). Here, we introduce a covalent, site-specific, rationally-directed enzyme immobilization technique utilizing non-canonical amino acid incorporation and the 2,3-dipolar Huisgen cycloaddition "click reaction" to enable complete enzyme immobilization orientation control.

Background

Candida antarctica Lipase B or CalB (EC 3.1.1.3) is an industrially relevant biocatalyst that enables a variety of biotransformations and has particular importance for organic synthesis (cite One Biocatalyst – many applications). CalB activity is not inhibited by the "click" reaction conditions and therefore is a compelling candidate for "click" reaction exploration (Schoffelen et al., 2008b). The enzyme is active as a single protein, and does not form multi-mers that might skew attachment results. Also, CalB has a simple, highly sensitive and widely accepted assay for activity, which is easily compared to standard, commercially available lyophilized enzyme (Sigma-Aldrich). A recent article described immobilizing CalB with unnatural amino acids, but used a global replacement method (Kiick et al., 2002; Schoffelen et al., 2008a). To avoid such invasive changes we site-specifically incorporated an alkyne-terminally functionalized unnatural amino acid (p-propargyloxyphenylalanine, pPa) at only the desired location for attachment.

4.2 Materials and Methods

Prepare Azide Functionalized Superparamagnetic Beads

Terminal amine group functionalized superparamagnetic M-270 Dynabeads were purchased from Invitrogen. The beads were then modified using a procedure adapted from literature to replace the amine functional groups with azide functional groups that could be used for click conjugation (Figure 4-1) (Punna et al., 2005). First, 5-azidopentanoic acid 4-nitrophenyl esters were synthesized through the addition of 5-azidopentanoic acid (3.5 mmol) and p-nitrophenol (3.7 mmol) to a solution with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (3.7 mmol) in CH_2Cl_2 at room temperature overnight.

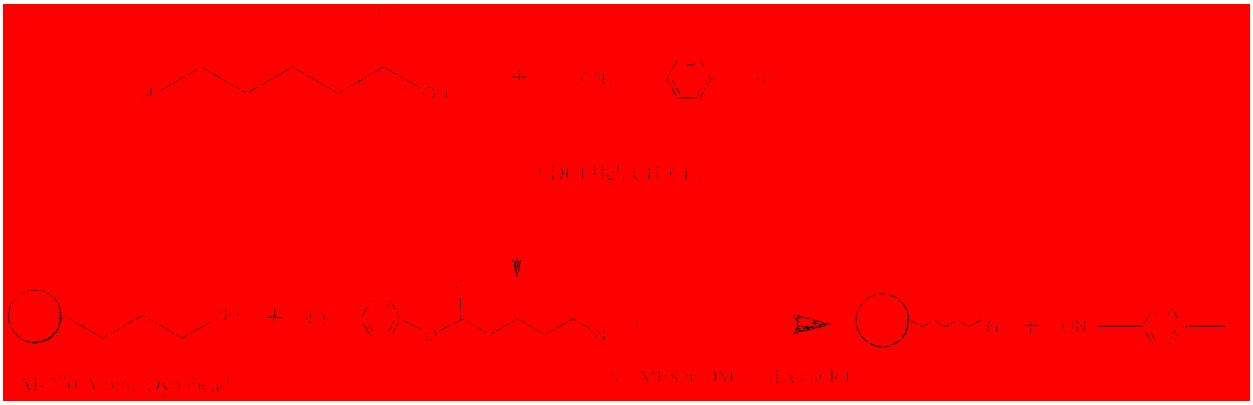


Figure 4-1: Organic synthesis steps used to modify amine-functionalized Dynabeads with terminal azide functional groups.

Using flash silica chromatography, the 4-nitrophenyl ester was purified and verified by Maldi-Tof mass spectrometry. Post-purification, the 4-nitrophenyl esters dried, dissolved in DMF, and added to a tube containing 2 ml of Dynabeads ($2x10^9$ beads per ml) in 5% MES buffer and stirred gently at room temperature for two days. The reaction mixture containing the functionalized beads was washed repeatedly with PBS buffer and transferred into a clean vial. Measurement of the p-nitrophenol absorbance using the optimal 412 nm wavelength was

performed and compared with a p-nitrophenol standard curve. A negative control reaction without beads was performed, with no change in absorbance at 412 nm.

The Dynabead manufacturer specifications note there are 0.1 mmol terminal amine groups per gram of dry beads and $7x10^{10}$ beads per gram dry beads. Relying on an assumption that the superparamagnetic beads are smooth spheres as shown in manufacturer provided literature, this suggests that there are approximately 35 terminal amines sites per nanometer squared. This high packing density conclusion was supported by the p-nitrophenol assays, which resulted in 95% conjugation or approximately 33 terminal azide groups per square nanometer. The density of the amine functionalized groups may be overestimated due to the unevenness and/or porosity of the polymer coating on the magnetic cores. The smooth sphere assumption is based upon images provided in manufacturer literature and used to clarify calculations.

4.3 Production of Candida antarctica Lipase B with Non-canonical Amino Acids

As discussed above, CalB was modified in a recent study for immobilization through the use of unnatural amino acids, but this was performed using a global replacement method that required the replacement of five amino acids (Schoffelen et al., 2008a). In this study, the five methionines were replaced by azidohomoalanine for conjugation purposes. For this enzyme, the modifications altered enzyme activity by >25%. However, for other enzymes, such extensive modification of the wild-type enzyme might have more substantial implications for enzyme activity, which would likely reduce its effectiveness as an alternative to traditional enzyme immobilization techniques. Moreover, this technique does not permit enzyme immobilization at rationally directed residues throughout the enzyme but rather limits immobilization to sites where a specific amino acid residue to be replaced may be found.

In contrast, the covalent enzyme immobilization technique that we have developed enables site-specific enzyme immobilization at residues throughout the enzyme while requiring only the change of a single amino acid residue through the substitution or insertion of a non-canonical amino acid. Through careful site-selection, the effects of enzyme modification can be minimized. This is supported by evidence in this study and more thoroughly in the lysozyme chapter below.

The non-canonical amino acid, p-propargyloxyphenylalanine, was site-specifically incorporated through the use of the E. coli-based cell-free protein synthesis as illustrated in Figure 4-2. Although a similar system was used previously to produce green fluorescent protein and is used later to produce lysozyme, significant differences between GFP, CalB, and lysozyme necessitated different approaches to protein production.

Unlike GFP, which may be produced in the E. coli cell-free protein production environment without significant cell extract modification, CalB is a eukaryotic yeast protein that suffers from significant solubility issues in an unmodified crude extract (Park et al., 2009a). The primary difficulty in CalB production results from the reductive nature of the unmodified E. coli cell-free extract. The reducing nature of the extract as well as the presence of many E. coli cysteine peptidases inhibits proper disulfide bond formation in CalB. As three disulfide bonds are necessary for CalB to fold correctly, obstacles to formation of any or all of the three bonds increase the risk of enzyme insolubility. Additionally, many non-canonical amino acids, such as the one utilized here, also suffer from solubility issues, increasing the difficulty of non-canonical amino acid insertion into a cell in vivo (Liu and Schultz, 2010). Fortunately for CalB protein production purposes, the versatility of the cell-free system enables us to overcome these

solubility issues and simplifies the addition of non-canonical amino acids for insertion into the protein production environment.

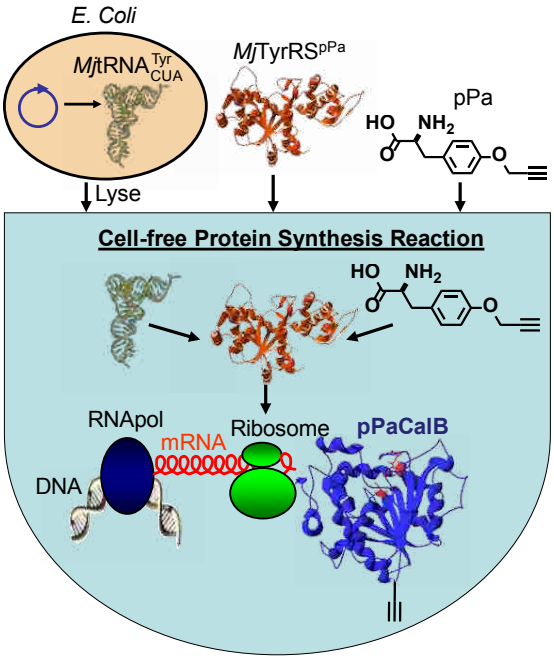


Figure 4-2: E. coli-based cell-free protein synthesis system modified for Candida antarctica Lipase B production.

4.3.1 Modifying the Cell-free Environment to Promote Disulfide Bonds

While in vivo protein production must be tempered to enable continued cell viability, cell-free protein synthesis systems permit protein production environments that may include extreme acidity or alkalinity, redox potentials, ionic strengths, or other conditions that would be otherwise inimical to cell survivability. Given the reducing nature of the crude E. coli extract, the literature described three key methods of increasing CalB solubility: 1) the addition of compounds to oxidize the crude cell extract, 2) the inhibition of the activity of endogenous E. coli cysteine peptidases using molecular inhibitors, and 3) the supplementation of the extract with chaperone proteins that enable the proper folding and formation of the lipase. These tests

were key to CalB protein production due to the fact that only 5% of the total CalB produced in our unmodified crude extract CFPS reactions were soluble.

For the first technique, prior work by the Kim group demonstrated that CalB solubility may be catalyzed by adding oxidized and reduced glutathione to the reaction solution in the ratio of 4 mM to 1 mM (Park et al., 2011a). We evaluated this recommendation in comparison to many other oxidized/reduced glutathione concentrations such as 3 mM/1 mM, 5 mM/1 mM, 8 mM/2 mM ratios and discovered that this was indeed the optimal reaction concentration (data not shown). This concentration was then used for all reported CalB reactions.

The second technique of inhibiting the activity of E. coli cysteine peptidases was used by preincubating our crude extracts with iodoacetamide. 2-iodoacetamide inhibits the activity of all cysteine peptidases. Based on preliminary tests, iodoacetamide provided slight increases in lipase activity, but did not prove as advantageous as hoped. This is likely due to the fact that the use of iodoacetamide requires a balancing act, as insufficient iodoacetamide fails to inhibit a sufficient percentage of cysteine peptidases, while an excess of iodoacetamide may inhibit disulfide bond formation through thiol interactions with the cysteines in the enzyme.

GroEL and GroES are chaperone proteins found in some strains of E. coli. The two proteins combine to form a complex that combine to encapsulate the folding protein, enabling proper protein folding (Figure 4-3). Although not specific to CalB, our tests demonstrated that the addition of a specially modified extract containing GroES and GroEL to the cell-free protein production reactions significantly improved protein solubility. After addition of GroES and GroEL, test results demonstrated that solubility increased from 5% of the produced protein to over 50% of all produced protein.

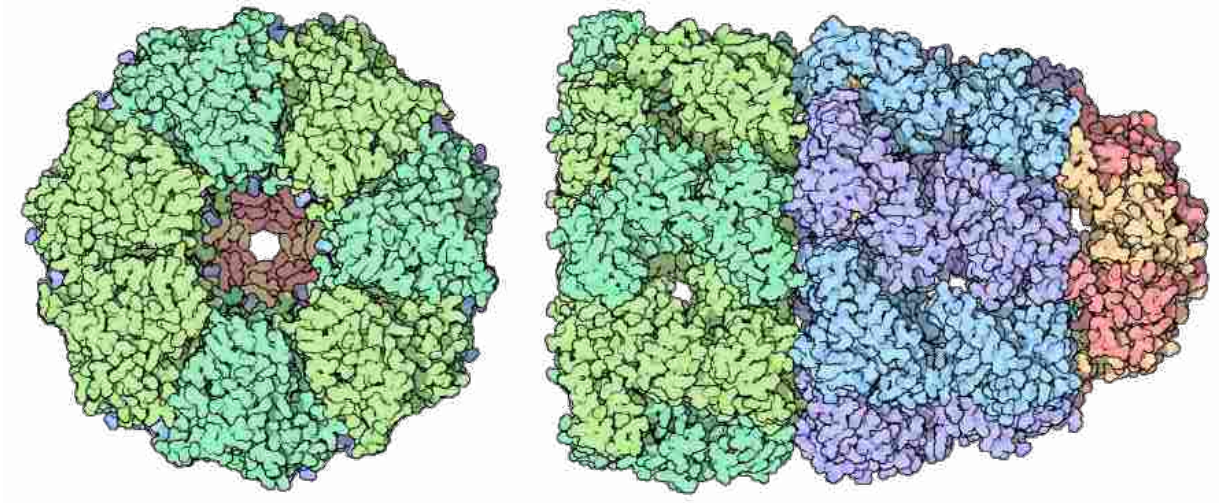


Figure 4-3: The GroEL and GroES Complex. It consists of the GroES cap (labeled in red and yellow) as well as the GroEL complex (labeled in blue, purple, and green) (Goodsell, 2002).

4.3.2 Selection of Non-canonical Amino Acid Incorporation Sites

Given that the goal of non-canonical amino acid incorporation was to test the effect on stability and activity of enzyme immobilization at various sites throughout the enzyme, we carefully selected three locations for study that varied greatly from one another. The three chosen locations were a site near the N-terminus, a site near the C-terminus, and a third site near the active site (Figure 4-4). The N-terminus site between Lys2 and Leu3 was chosen for study due to its importance as a region that is often heavily modified in enzymes. The C-terminus site, substituting pPa for Val196, was viewed as a site where the protein crystal structure demonstrates that the non-canonical amino acid would be on a loop that is readily available for enzyme immobilization and that is part of a long loop region that is less significant for protein structural stability.

Although the specially prepared GroEL and GroES extract greatly alleviated the issue of solubility, it increased the complexity of CalB preparation. The production of CalB with non-canonical amino acids requires the inclusion of special Methanocaldococcus jannaschii tRNA

and tRNA synthetases that are able to recognize and incorporate the non-canonical amino acid (Bundy and Swartz, 2010). After significant testing, it was observed that overexpression of both

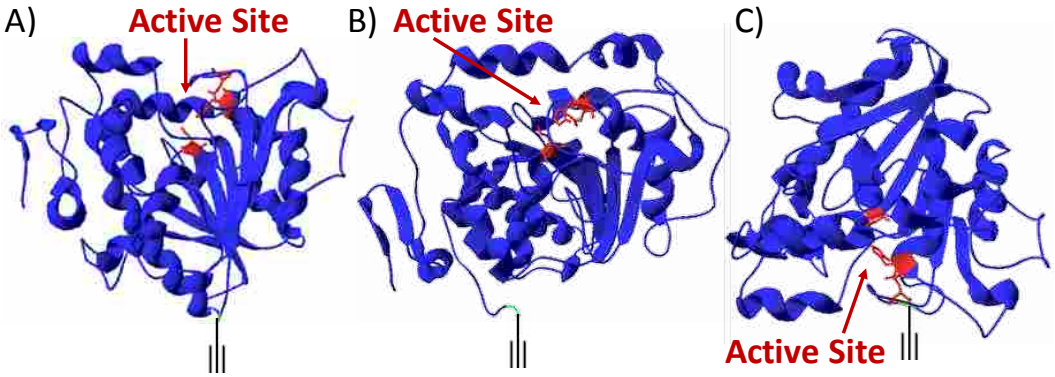


Figure 4-4: Non-canonical amino acid incorporation sites. A) pPa incorporation at the N-terminus between Lys2 and Leu3. B) pPa incorporation at the C-terminus as a substitution for Val196. C) pPa incorporation near the active site between Asn98 and Asn99.

GroES and GroEL proteins as well as the Methanocaldococcus jannaschii proteins in the same extract inhibited E. coli growth and reduced the viability of the cells and of the cell extract. Consequently, various proportions of GroEL/GroES cell extract combined with pEVOL extract containing the Methanocaldococcus jannaschii tRNA/tRNA synthetase pairs were tested to evaluate the optimal ratio for maximum CalB protein production (Figure 4-5).

The prototypical cell-free reaction, used for the production of GFP above and lysozyme below, utilizes a 15% volume to volume ratio of cell-free extract. However, given the solubility difficulties found in producing CalB, a mixture ranging from 25% v/v extract up to 45% v/v extract per batch reaction was tested. As the GroEL/ES extract v/v percentage increased from 15-20 percent, the amount of soluble protein produced increased significantly. Changing the pEVOL extract percentage from 15 up to 25 percent also led to substantial improvements in overall protein production, both soluble and insoluble (Figure 6-5). The combination of

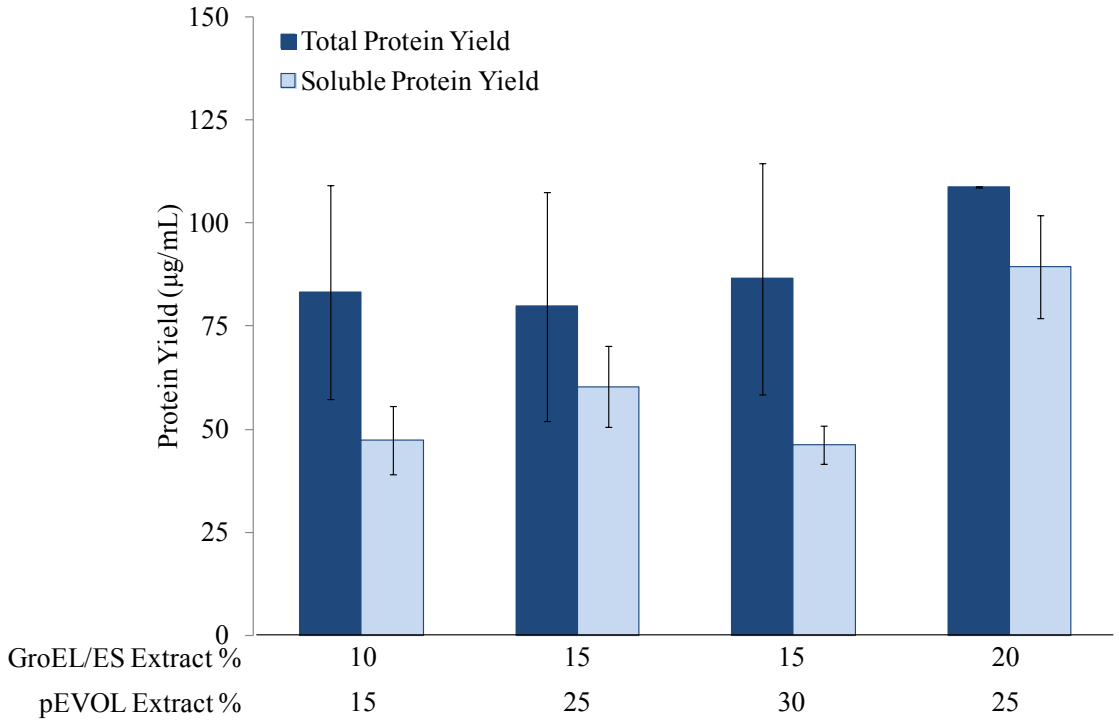


Figure 4-5: Wild-type CalB Production. Demonstrates total and soluble protein yields utilizing various ratios of GroEL/ES E. coli extract and pEVOL E. coli extract.

pEVOL extract with GroEL/ES E. coli extracts enabled recovery of greater than 75% of the total protein production in the soluble fraction.

The site-specific incorporation of non-canonical amino acids utilizing the amber codon replacement system described previously produces two types of proteins: full-length proteins with the non-canonical amino acid and truncated proteins without the amino acid (Smith et al., 2013). To quantify the amount of protein being produced and to ensure that full-length CalB was being produced by our system, we radiolabeled our proteins using ^{14}C leucine. These radio-labeled proteins were then run on an SDS-PAGE protein gel (NuPAGE, Invitrogen) and an autoradiogram was performed (Smith et al., 2013). Prior reports have demonstrated findings of less than 1% read-through when the non-canonical amino acid is not included in the protein production reaction and that greater than 99% of the produced protein is truncated in the absence of the unnatural amino acid (Wang et al., 2006). These results are supported by our findings

(Figure 4-6). As we had previous incorporated pPa in GFP, it is used as a control here. No full-length GFP or CalB is seen in the absence of pPa. The truncated product for the K2 CalB variant does not contain any leucines and thus was not visible on the autoradiogram. The N98 CalB variant contains only a few leucines and thus, its truncated product was also not observed.

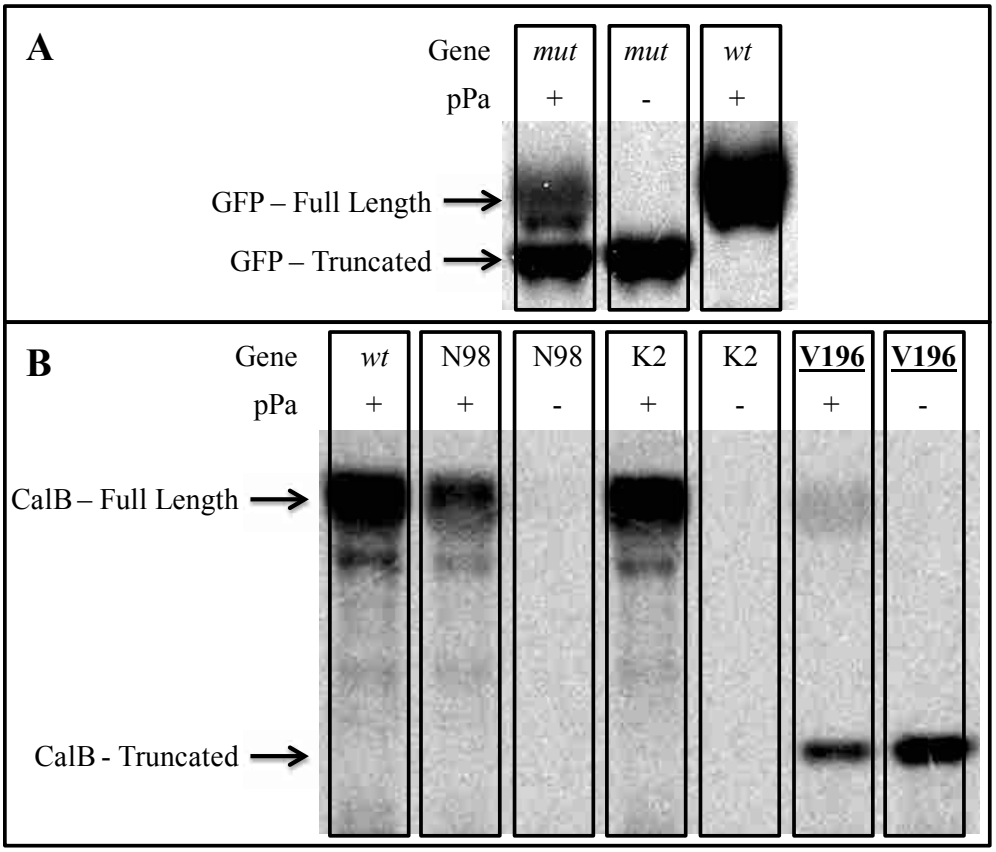


Figure 4-6: Autoradiograms of wild-type (wt) and pPa-incorporated GFP and CalB. A. An autoradiogram showing the effect of the addition and absence of pPa, with all observable protein truncated in the absence of the non-canonical amino acid at position 216. B. An autoradiogram showing full-length and truncated protein in the presence and absence of pPa. pPa is inserted after N98 and K2 and is substituted for V196.

Strep-tags were added onto the C-terminus of each CalB variant to enable purification of the full-length protein. Post-verification, strep-tactin columns (IBA Scientific, Goettingen,

Germany) were used to separate out the wtGFP, pPaGFP, wtCalB, K2 pPaCalB, N98 pPaCalB, and V196 pPaCalB using the manufacturer's protocols.

4.4 Covalent Site-specific "Click" CalB Immobilization on Superparamagnetic Beads

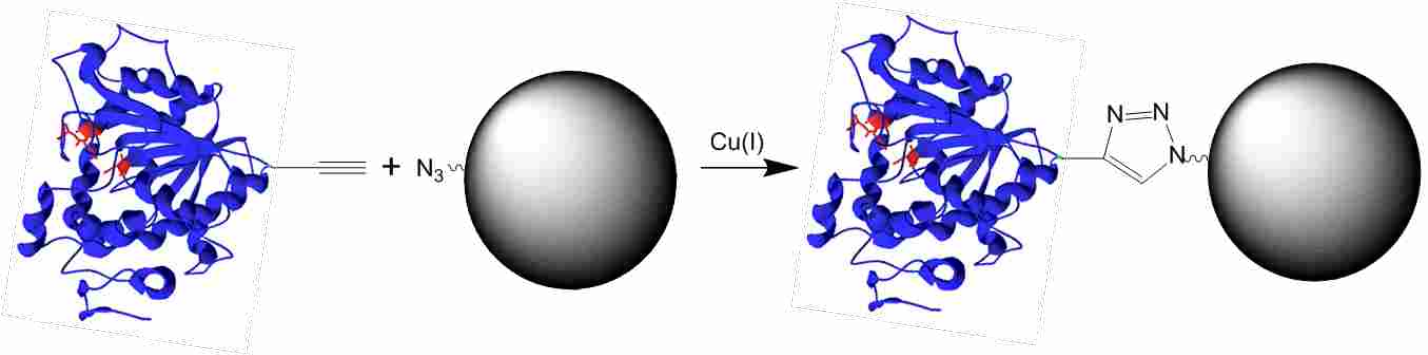


Figure 4-7: CalB Click Immobilization Scheme. The terminal alkyne group of the pPa enables covalent conjugation with azide functional groups on the surface of the superparamagnetic bead. Due to the site-specific incorporation of the amino acid, the enzyme's immobilization orientation is equivalent in each and every instance.

Our prior work optimizing click conjugation of Invitrogen's Alexa Fluor 555 (AF555) alkyne fluorophores and pPaGFP to superparamagnetic beads was adapted for application with CalB (Figure 4-7). Based on our success using 1 mM tetrakis(acetonitrile)copper(I) hexafluorophosphate (hereafter "Cu(I)Tet"), this copper catalyst was used again in the same concentration. However, due to the larger size of CalB compared with pPaGFP and AF555, the ratio of active site to enzyme was increased to prevent oversaturation of enzyme in the "click" reaction. For the "click" reactions, 0.06 nmol CalB was added to 1 mM Cu(I)Tet and beads equivalent to 10 nmol azide active sites were added and incubated in an anaerobic environment for 48 hours. Using liquid scintillation counting, click conjugation efficiency on the beads was 11%% ± 0.5% standard deviation (n=2).

4.5 In Solution and Immobilized CalB Variant Activity

The activity of the CalB variants were compared in solution and when immobilized. The CFPS-produced enzymes were compared to a commercially available CalB enzyme purchased from Sigma-Aldrich. The enzyme assay was performed based on a previously reported procedure which consisted of adding 0.05 mM p-nitrophenyl palmitate and ethanol (4% v/v) to a solution of 50 mM Tris-Cl buffer pH 8.0 (Park et al., 2009a).

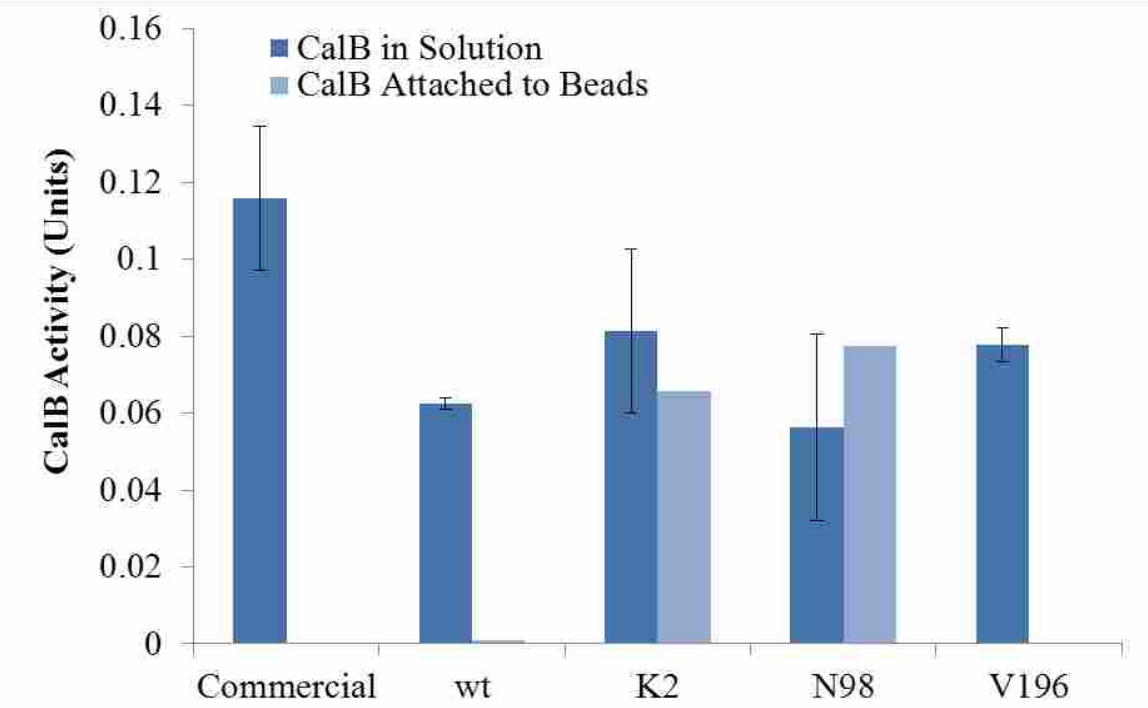


Figure 4-8: Specific Activity of Commercial CalB and CalB Variants. "Commercial" refers to commercial CalB purchased from Sigma-Aldrich. Wild-type CalB is labeled "wt," CalB with pPa inserted after K2 is labeled "K2," CalB with pPa inserted after N98 as "N98," and CalB with pPa replacing V196 is labeled "V196." Please note that all error bars denote standard deviation, n = 3. CalB attached to beads for K2 and N98 has activity measured n=1. The specific activity of V196 post "click" attachment was not measured.

Equivalent reaction mixtures were used for all variants, in solution or immobilized. The in-solution and immobilized enzymes were added to the reaction mixture for a total volume of 60 µl in trUView cuvettes (Bio-Rad, Hercules, CA). As CalB converts p-nitrophenyl palmitate to p-

nitrophenol, producing an absorbance change at 405 nm, the CalB variants were incubated at 37°C and the change in absorbance was measured in a Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA) after 30 minutes. Using a standard definition for activity units, a unit was defined as a 1 μmol increase in p-nitrophenol per minute per mg enzyme (Figure 4-8).

Measurement of the activity of CalB variants pre- and post-immobilization showed positive results. For variants K2 and N98, the change in activity post-immobilization was relatively minor and within a standard deviation. This suggested that CalB might be a positive candidate for covalent conjugation as it retained a significant amount of activity post-immobilization.

4.6 Conclusion: CalB Challenges, Lessons Learned

Although the process of producing the eukaryotic protein CalB and immobilizing it was a great learning process that proved very valuable for lysozyme immobilization studies below, it was one filled with many difficulties. It was very costly and time-consuming to produce sufficiently large and active concentrations of CalB in order to successfully immobilize it on the superparamagnetic bead surface. CalB yields in cell-free batch reactions were much lower than those of pPaGFP. Further, strep-tag purification of CalB seemed relatively inefficient, producing low post-purification yields. Given the low yields of CalB, which greatly increased the cost of CalB immobilization, the decision was made to switch to an enzyme that could more easily be produced at significant yields in the E. coli cell-free protein synthesis system. The decision to choose T4 bacteriophage lysozyme is discussed in the next chapter. Despite this, the valuable lessons learned about CalB production, solubility, and viability in the cell-free protein synthesis

system would prove to be an important component of a paper for which I was a coauthor, but which I do not include here (Smith et al., 2014)

5. Enhanced Enzyme Stability Through Site-Directed Covalent Immobilization

Much of this chapter (see Section 5.1 to 5.4) is an adaptation from an article titled "Enhanced Enzyme Stability Through Site-Directed Covalent Immobilization" that was recently accepted for publication by the Journal of Biotechnology. This work was developed and led by myself with the assistance of three undergraduate students, Mark Lindsay, Christopher Hutchings, and Christopher Werner. However, it is prefaced by a discussion not included in the paper establishing the rationale behind the selection of T4 bacteriophage lysozyme as an enzyme for study as well as an evaluation of the molecular modeling studies that have previously been performed on this enzyme. A further discussion of the lessons learned from comparing molecular modeling in silico techniques with in vitro techniques is provided in the next chapter.

5.1 Introduction

According to Environmental Protection Agency statistics, the U.S. alone contains more than 13,500 chemical manufacturing facilities providing shipments valued at over $555 billion annually (EPA, 2011). Unfortunately, traditional chemical manufacturing processes often require the use of toxic reagents, strong acids and bases, and have high energy costs while suffering from low product specificity. These processes produce more than 1.5 million tons of air pollution and over 10 billion pounds of solid waste annually, much of it toxic (EPA, 2011).

A more environmentally friendly alternative to conventional processes is the use of biocatalysts, or enzymes, to catalyze reactions (Bornscheuer et al., 2012). Enzyme use eliminates the need for many toxic reagents, enables reactions to be performed in aqueous conditions at ambient temperatures, and reduces the need for purification while providing high chemo-, regio-, enantio-, and stereo-specificity (Sheldon, 2007). These advantages have fueled a 7.5% annual growth rate in the industrial enzyme biocatalysis market from 1995 to 2012 (240%

total increase in market share) such that enzyme biocatalysis represented 10% of the global catalysis market in 2012 (Kirk et al., 2002; Milmo, 2012) However, enzymes have their own unique set of challenges including the following: enzyme production costs, enzyme stability, and enzyme recoverability and reusability (Wang et al., 2009b).

Enzyme immobilization provides a simple and elegant solution to these challenges. It enables recovery and reuse of the biocatalysts, significantly driving down the biocatalyst cost over many reactions. Currently, a number of different enzyme immobilization techniques are being used in industry such as enzyme adsorption, entrapment, and cross-linking (Sheldon, 2007). A particularly attractive method is attachment/adsorption of the enzyme to the surface of a carrier which commonly results in enhanced enzyme stability (Alonso et al., 2005; Anwar et al., 2007; López-Gallego et al., 2005; Mozhaev et al., 1990; Qiu et al., 2005; Rocchietti et al., 2004). Stability enhancements have been shown after attachment to many different carriers, examples include including magnetic beads (Qiu et al., 2005), mesoporous sol-gel glass (Wang et al., 2001b), silica nanospheres (Wang and Caruso, 2004), Sepabeads (Rocchietti et al., 2004), agarose (Rocchietti et al., 2004), and protein-coated microcrystals (Kreiner and Parker, 2005). Many different conjugation chemistries have been used, relying on amine, epoxy, maleic anhydride, glyoxal, biotin/streptavidin, and many other reactive functional groups for enzyme immobilization (Leckband and Langer, 1991; López-Gallego et al., 2013; Maeda et al., 2008; Mateo et al., 2007a; Mazi et al., 2006; Tasso et al., 2009; Zhang et al., 2011a) The chemical interactions used for surface attachment/adsorption may primarily be divided into three categories, Van der Waals-based adsorption, ionic adsorption, and covalent attachment (Sheldon, 2007).

Van der Waals enzyme adsorption techniques have been successfully used in industry,

but because these intermolecular forces are weak in comparison to ionic or covalent bonds, significant leaching of the enzyme from the support occurs over time (Zhao et al., 2006). Ionic adsorption improves retention over Van der Waals interactions, but leaching to a lesser extent is still common (Kirk and Christensen, 2002). Covalent immobilization is best suited for producing the strongest and longest lasting bonds between enzymes and surfaces, however it is the most prone to undesired surface-enzyme interactions resulting in activity loss (Barbosa et al., 2014; Kim et al., 2006a; Klibanov, 1979; Mateo et al., 2007b; Mozhaev et al., 1990). Traditionally, all of these techniques are limited in their inability to control the orientation at which the enzyme attaches/adsorbs to the surface to minimize undesired enzyme-surface interactions, maximize enzyme stability, and maximize active-site accessibility (Hernandez and Fernandez-Lafuente, 2011; Mateo et al., 2007b).

The development of technologies to enable site-specific enzyme immobilization at any desired residue on the protein would enable virtually all options. Prior modeling research has predicted that certain attachment sites can minimize undesired enzyme-surface interactions and optimize thermodynamic stability of immobilized proteins and enzymes (Friedel et al., 2007; Talasaz et al., 2006; Wei and Knotts, 2011b; Zhuoyun et al., 2009). Moreover, careful immobilization orientation control can minimize the effects of steric hindrance of the enzyme active site post-immobilization and provide greater understanding of the enzyme-surface interactions.

Previous research demonstrating covalent site-specific immobilization techniques have suffered from significant limitations in potential immobilization locations, such as the N- or C-terminal regions (Smith et al., 2005; Tominaga et al., 2004). Other site-specific immobilization technologies have enabled immobilization at many potential residues, but rely on noncovalent

techniques (Kalia et al., 2007; Lim et al., 2014; Seo et al., 2011). To our knowledge, no covalent site-specific immobilization method has been previously demonstrated enabling immobilization at residues throughout the enzyme while only requiring a single substitution or insertion. In this paper, we seek to address the need of a covalent site-specific system enabling immobilization at potential residues throughout the enzyme to prevent enzyme leaching and also analyze the impact of immobilization orientation on enzyme stability.

Here, we report our PRECISE system's (Protein Residue-Explicit Covalent Immobilization for Stability Enhancement) (Smith et al., 2013) effective application for covalent, site-specific immobilization and increased stability of enzymes. This system facilitates easy, non-invasive enzyme recoverability and reusability, testing of enzyme activity at different immobilization sites, and comparisons of enzyme stability when attached at different orientations. These benefits are accomplished through the use of amber-codon-substitution to enable rationally-directed non-canonical amino acid incorporation for site-specific enzyme immobilization using the covalent, biocompatible, and bioorthogonal Huisgen 1,3-dipolar cycloaddition reaction (Boyce and Bertozzi, 2011; Hong et al., 2010; Smith et al., 2013; Young et al., 2010). The results from these tests indicate that enzyme immobilization orientation plays a significant role in enzyme activity and stability and that meaningful stability enhancements above and beyond that provided by immobilization alone can be found through careful immobilization orientation control.

5.2 Selection of Model Enzyme for Study

Lysozyme was chosen for study because of its well-characterized folding and denaturation pathways (Peng and Li, 2008; Wildegger and Kiefhaber, 1997). T4 bacteriophage lysozyme is a molecule that is 164 residues in length, and is classified as an orthogonal-bundle

protein that is primarily alpha in nature. It is a multistate folding protein that follows a slow-folding pathway 20% of the time, leading to a stable intermediate (Kiefhaber, 1995). In the remaining 80% of folding opportunities, the protein follows a quick-folding pathway where it folds quite rapidly and does not form a stable intermediate (Kiefhaber, 1995). However, the presence of a stable intermediate has complicated understanding of lysozyme folding. Due to computational limitations, molecular modeling of surface-protein and surface-enzyme interactions have relied until recently on two-state folding mechanism models for proteins (Wei and Knotts, 2011b). Models that rely on two-state folding mechanism are sufficient for some proteins (Zwanzig, 1997) that exist primarily in folded or unfolded states but lack the ability to effectively evaluate proteins with complicated transition states.

Recent improvements in molecular modeling systems now enable the study of proteins with multiple folding states, enabling the evaluation of enzymes such as lysozyme with a stable intermediate. These recent improvements enabled the recent evaluation of the effects of tethering lysozyme to a surface (Wei and Knotts, 2011b). This molecular modeling of T4 lysozyme relied on a course-grain model with a short-range repulsive surface that evaluated the interaction of the enzyme with the surface only when a single residue came into close proximity with the surface (Wei and Knotts, 2011b). Although this in silico approach is greatly simplified, it enables molecular modeling studies of a system very similar to the PRECISE site-specific system described herein where our protein or enzyme of interest is immobilized at a single site or residue, and the interaction between the protein and surface occurs primarily in this region.

Building upon this molecular modeling approach with a real-world experimental study of lysozyme is necessary because even the most current of molecular modeling technologies relies on significant simplifications of many factors that can affect enzyme stability such as

interactions between the enzyme and the solvent, the interactions between the solvent and the surface, interactions between neighboring proteins, interactions between neighboring surfaces, and any and all combinations of all of these interactions. Conclusions about the thermodynamic stability of a single protein interacting with a single surface may or may not prove equally true for industrial applications that rely on the immobilization of many enzymes on a surface with the many combinations of interactions discussed above. In contrast, experimental validation of conclusions made from molecular modeling systems may demonstrate that despite the simplifications made in molecular modeling, these thermodynamic models provide relatively robust systems for analysis and provide a strong foundation for future experimental testing.

Here, Shuai et al. predicted that the immobilization of lysozyme at residue 91, a leucine, would provide a particularly thermodynamically stable conformation, enhancing lysozyme stability above that of its unimmobilized form found in solution (Wei and Knotts, 2011b). In this simulation, the researchers observed an interesting result, namely that immobilization of lysozyme at this residue eliminated the stable intermediate as one of the possible folding states, converting lysozyme into a two-state folding protein. This stable intermediate involves the separation or breaking away of the first, structurally important alpha helix from the rest of the enzyme. The elimination of this stable intermediate reduces the number, types and stability of misfolded and incompletely folded lysozyme states, thus, significantly increasing the overall stability of the lysozyme when tethered at site 91. Based on this finding, leucine 91 was carefully chosen as one of the sites for examination in our study.

As a noteworthy change, however, our study goes beyond the examination of the previous molecular modeling study in that we evaluate enzyme stability not only within the context of enzyme stability in a heat and temperature context but stability in the presence of

chemical denaturants, notably urea. Due to the marked differences in destabilizing effects played by chemical denaturants as opposed to the effects caused by temperature, this was also deemed a fruitful area of study.

5.3 Materials and Methods

5.3.1 Cloning

A T4 lysozyme variant (Plasmid 18111) with two mutations, C54T and C97A, but equivalent activity and stability to the wild-type lysozyme was acquired from Addgene (Cambridge, MA) (Matsumura and Matthews, 1989). This T4 lysozyme variant simplified cell-free protein synthesis (CFPS) because it eliminated the need to alter the environmental redox potential to promote disulfide bond formation (Matsumura and Matthews, 1989). As an additional benefit, the denaturation pathway of this variant has been well-characterized (Peng and Li, 2008).

For protein purification purposes, a strep-tag (consisting of the eight amino acids Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) was added to the C-terminus of the cysteine-free T4 lysozyme using the Quikchange II mutagenesis protocol (Agilent Technologies, Santa Clara, CA). This T4 lysozyme is hereafter referred to as the "standard" lysozyme. Six sites were selected for modifications to enable non-canonical amino acid incorporation at each site and immobilization at that site. These sites were T21, K35, N53, L91, K135, and K162 and the Quikchange II mutagenesis protocol was used to substitute the amber stop codon at each location (Figure 5-1A).

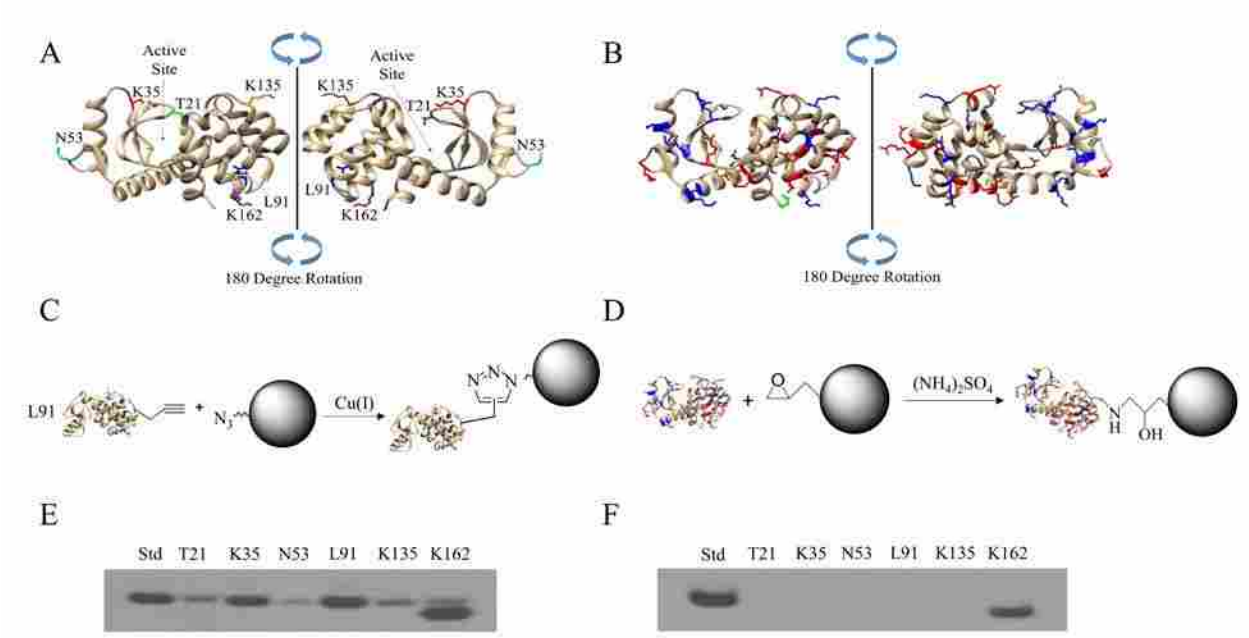


Figure 5-1: Site-specific vs. Nonspecific Covalent Immobilization. A. Location of six different non-canonical amino acid incorporation sites tested as potential immobilization sites. B. T4 lysozyme structure showing all possible nonspecific immobilization attachment sites at arginines (red), lysines (blue), and the N-terminus (green). C. The PRECISE System attachment scheme utilizing non-canonical amino acid incorporation and the Huisgen 1,3-dipolar cycloaddition ("click" reaction) to site-specifically covalently immobilized enzymes on superparamagnetic beads. D. Nonspecific covalent immobilization scheme using epoxy-functionalized beads treated to bind to reactive amines. E. Autoradiogram demonstrating lysozyme variant protein production when the non-canonical amino acid is added to the cell-free protein synthesis reaction. F. Autoradiogram demonstrating that full-length lysozyme variant production was not detected when the non-canonical amino acid was not added. Note the truncation band for K162 is due to termination of the protein at the amber stop codon, which is only 10 codons from the C-terminus. As all produced proteins were subjected to a strep-tag purification process where such strep-tag was located on the C-terminus of the lysozyme, only full-length proteins were recovered and used for further experiments. (Wu et al., 2015).

5.3.2 Cell-free Protein Synthesis

The Escherichia coli cell-free protein synthesis system is a protein production technology particularly valuable for its scalability (Zawada et al., 2011). Previously researchers have demonstrated equivalent protein production from the 100 µl to the 100 liter scale (Zawada et al., 2011). Here, CFPS reactions for protein production were performed as described previously

(Smith et al., 2013). The cell-free extract was prepared as described previously (Shrestha et al., 2014) and was produced from a BL21 Star™ (DE3) E. coli strain purchased from Invitrogen (Carlsbad, CA) harboring the pEVOL-pPrF plasmid, a kind gift from Peter Schultz, (Scripps Research Institute). For cell-free reactions for lysozyme variants incorporating non-canonical amino acids, 2 mM p-propargyloxyphenylalanine (pPa) and 3 mg/ml pPa specific Methanocaldococcus jannaschii pPa tRNA-synthetase were additionally added (Smith et al., 2013). Post-cell free reaction, lysozyme was purified from the CFPS reaction using Strep-Tactin Superflow columns (IBA Life Sciences, Gottingen, Germany). The lysozyme variants were quantified as described below.

5.3.3 Protein Yields

The yields for the total and soluble protein were calculated using liquid scintillation as described previously (Smith et al., 2013). Full-length protein production was verified by running sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by autoradiography as previously reported (Smith et al., 2013). In all reactions where the non-canonical amino acid was not added, all T4 lysozyme proteins were truncated and no full-length protein was observed (Figure 5-1F). In contrast, where the non-canonical amino acid was added, full-length T4 lysozyme was produced (Figure 5-1E). Once full-length protein production was verified, soluble proteins were recovered from the supernatant post-centrifugation at 13,000 x g for 15 minutes and purified by strep-column purification per the manufacturer's specifications (IBA Life Sciences, Goettingen, Germany).

5.3.4 Enzyme Immobilization on Superparamagnetic Beads

Nonspecific covalent lysozyme immobilization was performed using M-270 Epoxy Dynabeads (Life Technologies, Carlsbad, CA). (Figure 5-1D). The manufacturer specified

protocol was used for immobilization. This immobilization method enables covalent immobilization at all arginines, lysines, and the N-terminus (Figure 5-1B). 200 µl reactions were used with 300 µM of available epoxy sites and 3 µM lysozyme. The immobilization of the enzyme was verified by liquid scintillation counting of C^{14} leucine-radiolabeled lysozyme. For site-specific attachment, the Huisgen 1,3-dipolar "click" reaction was used (Figure 5-1C). Click reaction conditions consisted of 2 mM $CuSO_4$, 10 mM sodium ascorbate, 4 µM THPTA, 300 µM of azide functionalized M-270 Amine Dynabeads as discussed previously, and 3 µM of lysozyme with a total reaction volume of 200 µl (Smith et al., 2013). The reaction was added into a 1.5 ml microcentrifuge tube and rotated end over end for 24 hours at 4ºC. Enzyme immobilization was verified by liquid scintillation counting of C^{14} leucine-radiolabeled lysozyme.

5.3.5 Purification of Lysozyme Immobilized Dynabeads

Immobilized lysozyme was purified using the following procedure. Microcentrifuge tubes containing reaction mixtures were centrifuged and placed on a Dynaspin Magnet (Life Technologies) for 8 minutes to ensure that all beads were firmly pulled to the microcentrifuge tube sidewalls by the magnet. All reaction reagents except the beads were removed by careful pipetting. Next, careful washing of the beads to remove noncovalently immobilized lysozyme was performed and the buffer was exchanged four times repeating the following procedure. First the beads were resuspended with 100 µl of PBS buffer at 4ºC and vigorous vortexing. After vortexing, the beads were incubated for 15 minutes at 4ºC. Samples were then returned to the DynaMag Spin Magnet and the supernatant removed by pipetting. After the final washing step was complete, the beads were resuspended and subsequently stored at 4ºC in the Enzchek Lysozyme Assay reaction buffer (Life Technologies, Carlsbad, CA). 1x Enzchek Lysozyme

Activity Assay reaction buffer (hereafter referred to as "assay reaction buffer") consists of 0.1 M sodium phosphate, 0.1 M sodium chloride, and 2 mM sodium azide at a pH of 7.5.
Liquid scintillation counting demonstrated that on average 7.7±0.96% of the lysozyme added to the immobilized reaction was conjugated on the superparamagnetic beads. Using surface area data provided by the manufacturer and conservatively assuming that lysozymes would horizontally orient in the most space-saving fashion, it was estimated that at the average coupling efficiency of 7.7%, immobilized lysozymes would cover 53.9% of the surface area of each individual superparamagnetic bead. Thus, higher conjugation efficiencies were likely inhibited by steric hindrances.

5.3.6 Lysozyme Activity Assay

A standard commercial lysozyme activity assay, the Enzchek Lysozyme Assay Kit (Life Technologies) was used to determine enzyme activities using the following protocol: 2.75 µg of Micrococcus lysodeikticus substrate was suspended in assay reaction buffer for a total volume of 50 µl and incubated for 15 minutes at 37ºC. The 50 µl of soluble substrate was added to 50 µl of covalently immobilized Dynabeads suspended in assay reaction buffer or 50 µl of unimmobilized lysozyme suspended in assay reaction buffer. Each 100 µl reaction was added into a Corning Costar 3915 (Corning, NY) black fluorescent 96 well plate. Fluorescein fluorescence released by lysozyme activity was measured per the assay kit instructions for 60 minutes using a Biotek Synergy MX monochromator set to 494 nm excitation and 518 nm emission wavelengths. The specific activity of each lysozyme was quantified after the 60 minute time point and normalized to the specific activity of the standard unimmobilized and untreated lysozyme. The average concentration of the immobilized and free enzyme concentrations were assessed for activity at 6.1±1.5 µg/ml and 22.1±5.1 µg/ml respectively.

5.3.7 Freeze Thaw Cycles

50 µl of aliquots of site-specifically bead-immobilized lysozyme, nonspecifically bead-immobilized lysozyme and unimmobilized lysozyme were tested for activity prior to freeze thaw by using the lysozyme activity assay described above. After testing and washing, the lysozyme immobilized beads and a fresh 50 µl aloquot of unimmobilized lysozyme were added to a 96 well plate and carefully sealed to prevent evaporation. The 96 well plate was stored at -80ºC for 20 minutes and then immediately placed at 25ºC for 20 minutes, a change of 105ºC. After the completion of this cycle, the plate was returned to -80ºC for the beginning of the second cycle. Three total freeze thaw cycles were performed. Post-freeze thaw cycles, the lysozyme activity assay was performed to determine change in lysozyme activity after freeze thaw.

5.3.8 Urea Incubation

50 µl of aliquots of site-specifically bead-immobilized lysozyme, nonspecifically bead-immobilized lysozyme and unimmobilized lysozyme were tested for activity prior to urea denaturation by using the lysozyme activity assay. The assay was slightly modified by the addition of an extra 50 µl of assay reaction buffer (total volume of 150 µl instead of 100 µl). The lysozyme immobilized beads were then washed and stored in microcentrifuge tubes. 50 µl of fresh unimmobilized lysozyme were also added to microcentrifuge tubes. Urea in reaction buffer was added to both sets of lysozyme for a final concentration of 2 M urea and a final volume of 100 µl. Each reaction was then incubated in urea at 37ºC for 30 minutes. After urea incubation, 2.75 µg of Micrococcus lysodeikticus substrate solubilized in 50 µl total volume of reaction buffer was added to each urea lysozyme set, creating a total of 150 µl for each reaction. Each reaction was added into a 96 well plate and tested for fluorescence using the same excitation and emission wavelengths described above.

5.4 Results and Discussion

5.4.1 Utilizing Non-Canonical Amino Acid Incorporation to Enable the PRECISE System

The PRECISE system enables site-specific, orientation-controlled, covalent immobilization of enzymes onto a chosen carrier or surface. Through the use of rationally directed, site-specific amino acid incorporation, we have previously shown that the model reporter protein GFP's stability significantly improved when subjected to denaturing conditions (Smith et al., 2013). Here, we report for the first time the covalent, site-specific immobilization of enzymes using the PRECISE system. As described in the Material and Methods section, T4 lysozyme was used as a model enzyme. The PRECISE-immobilized T4 lysozyme activity and stability under denaturing conditions is directly compared to the stability of T4 lysozyme immobilized using traditional covalent immobilization techniques. For all immobilizations, superparamagnetic beads were employed as the enzyme carrier due to their nontoxic and biocompatible nature and facilitate non-invasive enzyme recovery (Cao et al., 2012; Dyal et al., 2003; Lei et al., 2007; Yu et al., 2012). The standard lysozyme, a cysteine-free T4 lysozyme variant from Addgene, was used as a template to create six different lysozyme variants each with a single pPa non-canonical amino acid insertion (Figure 5-1A). pPa was selected as a non-canonical amino acid enabling a click conjugation reaction (Figure 5-1C). Variants T21pPa and K35pPa were chosen for their proximity to the activity site with T21pPa directly next to the active site and K35pPa in the same region as the active site. N53pPa, K135pPa, and K162pPa were chosen as sites that had high surface accessibility and were far from the active site region and for their presence in less structurally important loop regions. L91pPa was selected due to prior work suggesting that it would be an optimal, thermodynamically stable tethering site (Wei and Knotts, 2011a) (Figure 5-1A).

The nonspecific covalent enzyme immobilization technique targeting all arginines, lysines,

and the N-terminus in the enzyme was chosen because it enabled immobilization at any one of many sites distributed relatively equally in the enzyme, near and far from the active site, surface accessible or buried, near or on protein loops, alpha helices, and beta sheets. Given the 26 potential attachment residues (13 arginines, 12 lysines, and the N-terminus), approximately 1 in every 6 residues was a potential immobilization site (Figure 5-1B, 5-1D).

Auto-radiograms were performed to ensure that full-length T4 lysozyme variants containing the non-canonical amino acid were produced when the non-canonical amino acid pPa was added (Figure 5-1E) and that full-length T4 lysozyme was not produced when the non-canonical amino acid was omitted in the CFPS reaction (Figure 5-1F).

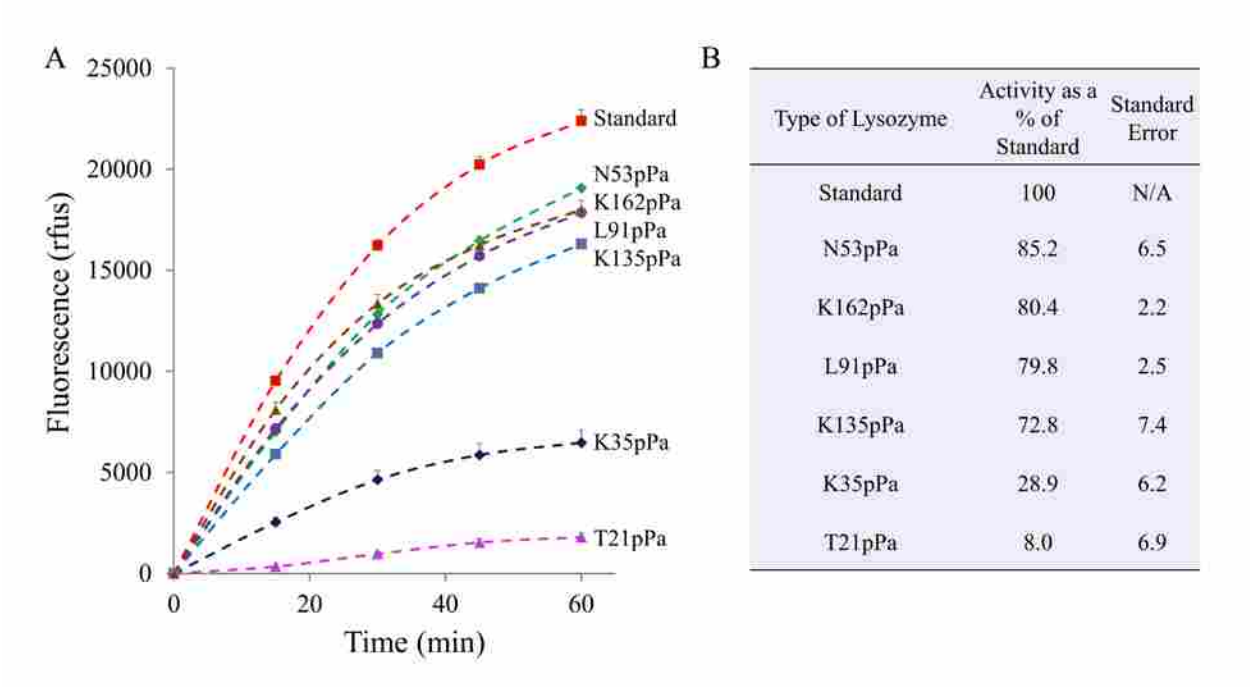
The post-protein purification yield for the standard T4 lysozyme ranged from 0.6 mg to 0.7 mg per 1 ml batch cell-free protein synthesis (CFPS) reaction. CFPS reactions ranging from the 15 µl to the 1 ml scale had similar protein production yields ranging from 0.6 mg to 0.7 mg per 1 ml batch reaction. Because the amber codon substitution system is more complex, the yields for the lysozymes with non-canonical amino acids were lower. The post-purification yield for T21pPa, K35pPa, and L91pPa lysozymes ranged from 0.25 mg to 0.3 mg per 1 ml batch reaction. For N53, K135, and K162, protein yields were typically less than one-third as productive, ranging from 0.04 mg to 0.1 mg per 1 ml batch reaction.

5.4.2 Activity of the T4 Lysozyme Variants

The insertion of the non-canonical amino acid, pPa, had divergent effects on the activity of each lysozyme variant dependent upon the location of the mutation at each of the six rationally engineered sites (Figure 5-2). The lysozyme variants immediately next to the active site (T21pPa) and near the active site (K35pPa) suffered the most dramatic decrease in activity, with the lysozyme variant T21pPa retaining less than ten percent of the activity of the standard lysozyme.

Given the proximity of the non-canonical amino acid to the active site, these effects were expected and consistent with previous research that report the effect of amino acid mutations near the active site (Greenwald et al., 1999). The three variants that were chosen for their surface accessibility and distance from the active site, N53pPa, K135pPa, and K162pPa, maintained much higher activity, ranging from 70 percent to 85 percent of the activity of the standard lysozyme. The final variant, L91pPa, showed activity in solution that placed it in the middle of the range between the three variants N53pPa, K135pPa, and K162pPa that were further from the active site. These differences in enzyme activity post-unnatural amino acid insertion demonstrate the importance of careful selection of the insertion site.

This data supports avoiding the active site region and choosing surface exposed and flexible areas for non-canonical amino acid incorporation. However, avoiding active sites and choosing surface exposed sites for non-canonical amino acid incorporation becomes difficult in instances where the protein of interest's structure remains unsolved. Given that the Protein Data Bank's approximately 92,000 solved structures remains a small fraction of all known protein sequences, the continued acquisition of data on the effect of non-canonical amino acid incorporation sites on activity and structure as well as the development of better experimentally validated simulation software is needed to realize the full potential of non-canonical amino acid applications.



B

Type of Lysozyme	Activity as a % of Standard	Standard Error
Standard	100	N/A
N53pPa	85.2	6.5
K162pPa	80.4	2.2
L91pPa	79.8	2.5
K135pPa	72.8	7.4
K35pPa	28.9	6.2
T21pPa	8.0	6.9

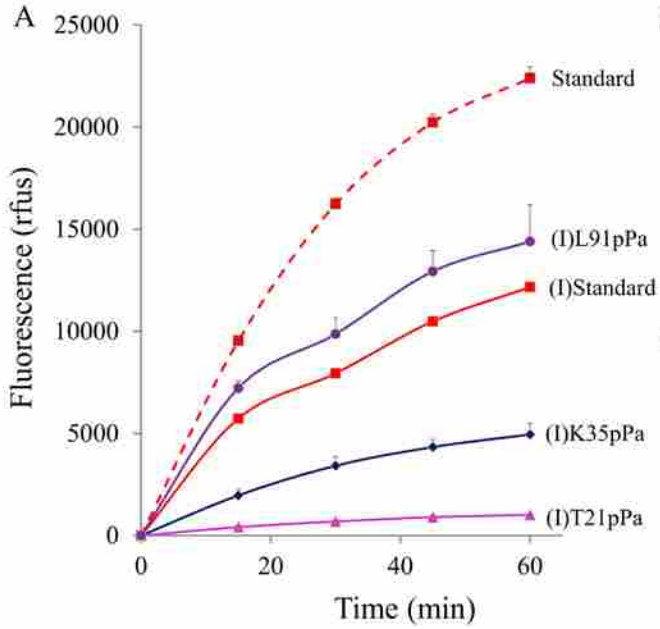
Figure 5-2: T4 Lysozyme Specific Activity. A. The activity of each lysozyme measured for 60 minutes at 37ºC. The activity shown is normalized for enzyme concentration and thus is a measure of the specific activity of each lysozyme. B. The specific activity of each lysozyme normalized to the specific activity of the standard lysozyme. (Wu et al., 2015).

5.4.3 Effect of Lysozyme Immobilization on Lysozyme Activity

Due to the consequences of steric effects and steric hindrance in particular, the orientation of the immobilized enzyme can have a significant effect on enzyme activity. To test these effects, lysozyme variants were covalently immobilized on superparamagnetic beads at the non-canonical amino acid site using PRECISE technology (Smith et al., 2013) (Figure 5-1C). For scaled up production and immobilization the lysozyme variants T21pPa, K35pPa, and L91pPa were specifically chosen because of high protein expression yields in the E. coli-based cell-free protein synthesis system and their representation of a location at the active site, a location in the proximity of the active site, and a location away from the active site which is also the best location predicted by simulation (Wei and Knotts, 2011a). The specific activity of each immobilized variant was measured and compared to covalent nonspecifically immobilized standard lysozyme (Figure 5-3).

The activity of standard lysozyme prior to immobilization was used as a conservative control for comparison as it had significantly higher activity than any other lysozyme variant prior to immobilization.

A



B

Type of Lysozyme	Immobilized	Activity as a % of Standard	Standard Error
Standard		100	N/A
Standard	X	54.3	1.5
L91pPa	X	64.3	7.4
K35pPa	X	22.1	6.7
T21pPa	X	4.5	3.2

C

Type of Lysozyme	Immobilized	% Change in Activity	Standard Error
Standard	X	-45.7	1.5
L91pPa	X	-19.4	7.5
K35pPa	X	-23.4	10.4
T21pPa	X	-43.6	7.7

Figure 5-3: T4 Lysozyme Specific Activity Post-Immobilization. A. The specific activity of each lysozyme variant measured for 60 minutes at 37ºC. The activity shown is normalized for enzyme concentration and thus is a measure of the specific activity of each lysozyme. The (I) symbol is added to signify each type of lysozyme that is immobilized. B. The specific activity of each immobilized lysozyme normalized to the unimmobilized standard lysozyme. C. Percentage change in lysozyme activity post-immobilization. (Wu et al., 2015).

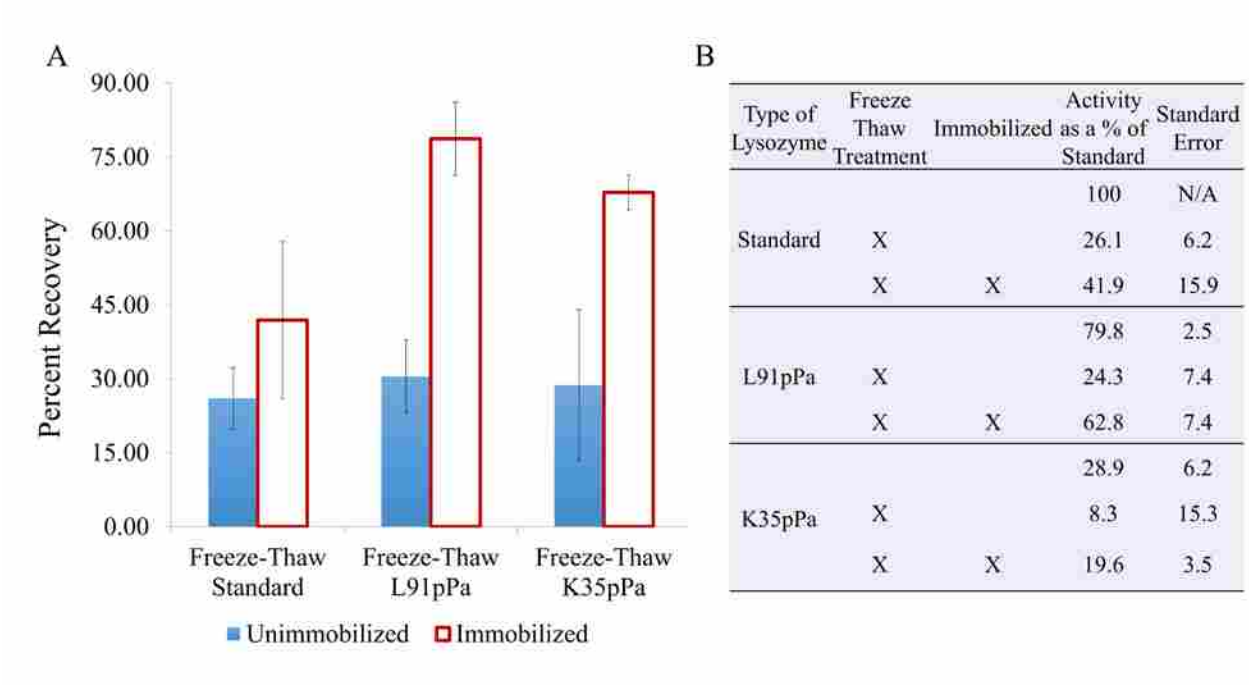
The distinct changes in enzyme activity for each lysozyme variant post-immobilization strongly suggest that immobilization orientation has a significant effect on enzyme activity (Figure 5-3). As initially predicted, the immobilization next to active site (T21pPa) resulted in a large decrease in specific activity (44% drop). Nonspecifically immobilizing the standard lysozyme also resulted in a similar, large decrease in specific activity in percentage terms (46% drop), although it maintained higher activity in absolute terms than the T21pPa immobilized variant. In contrast, the remaining two site-specifically immobilized variants, L91pPa and K35pPa, exhibited

much smaller drops in specific activity (19% and 24% drop respectively). Consequently, L91pPa had the highest absolute activity of all the immobilized lysozyme types post-immobilization (Figure 5-3). Thus, these results suggest that the advantages of immobilization orientation control in the demonstrated system can outweigh the costs in enzyme activity due to the incorporation of the non-canonical amino acid.

5.4.4 Effect of Lysozyme Immobilization Orientation on Lysozyme Activity Retention Post-Freeze Thaw Cycling

Following immobilization, we evaluated the effect of immobilization orientation on activity after subjecting the enzymes to denaturing conditions. For this evaluation immobilized K35pPa, L91pPa and standard lysozyme variants were used. The immobilized T21pPa lysozyme variant was not used due to its very low activity close to background. The immobilized K35pPa variant was included in the stability experiments as it had higher activity than the T21pPa variant and enabled us to examine the effect of near-active site immobilization on post-immobilization lysozyme activity. Given that enzymes are commonly frozen for storage and later thawed for both industrial and research purposes, with the freezing and thawing process producing significant changes in buffer and salt concentrations, which can cause denaturation in the absence of a cryoprotectant, freeze thaw cycles were utilized to assess the role that immobilization orientation plays in promoting or weakening enzyme stability (Pikal-Cleland et al., 2000).

After subjecting the lysozyme variants to three freeze thaw cycles of -80ºC to 25ºC in the absence of a cryoprotectant, the standard lysozyme, K35pPa, and L91pPa variants all suffered a significant decrease in activity, with all enzymes retaining only 20 to 30 percent of their pre-freeze thaw activity (Figure 5-4).



B

Type of Lysozyme	Freeze Thaw Treatment	Immobilized	Activity as a % of Standard	Standard Error
Standard			100	N/A
	X		26.1	6.2
	X	X	41.9	15.9
L91pPa			79.8	2.5
	X		24.3	7.4
	X	X	62.8	7.4
K35pPa			28.9	6.2
	X		8.3	15.3
	X	X	19.6	3.5

Figure 5-4: Post-Freeze Thaw Stability. Each lysozyme was subjected to three freeze thaw cycles. Each cycle consisted of 20 minutes at 80°C followed by 20 minutes at 25°C. A. Percent of lysozyme activity retained post-freeze thaw. B. Normalized lysozyme activity standardized to the untreated and unimmobilized standard lysozyme. (Wu et al., 2015).

In contrast, all of the immobilized enzymes, specific and non-specific retained significantly greater activity, with far greater activity retention seen with the K35pPa and L91pPa (~70-80% retention) site-specifically attached lysozyme variants compared to nonspecifically immobilized standard lysozyme (42% retention). In absolute terms, immobilized L91pPa was 141% more active than the unimmobilized standard lysozyme post-freeze thaw and 50% more active than the nonspecifically immobilized standard lysozyme (Figure 5-4). Thus, although traditional nonspecific enzyme immobilization affords increases in enzyme stability, such stability improvements can be significantly increased through an attachment that enables enzyme immobilization orientation control.

5.4.5 Effect of Lysozyme Immobilization Orientation on Lysozyme Activity Retention Post-Urea Incubation

The mechanism of urea denaturation is believed to be caused by direct urea-protein interactions and/or by perturbation of solvation environment around the protein by urea, increasing solvation of hydrophobic groups (Hua et al., 2008). Urea denaturation thus acts in a very different denaturation process than freeze thaw cycles, making it another useful tool for evaluating stability.

Similar to the data obtained after freeze thaw denaturation, site-specifically immobilized K35pPa and L91pPa lysozyme variants retained a higher percentage of activity than the nonspecifically immobilized standard lysozyme and all unimmobilized lysozymes. All unimmobilized lysozymes lost the majority of their activity retaining approximately 20 to 40 percent of their pre-urea activity (Figure 5-5). Similar to the freeze thaw denaturation, both of the site-specifically immobilized lysozyme variants retained more than 70% of their pre-urea incubation activity while the nonspecifically immobilized standard lysozyme lost 67% of its pre-urea activity. On an absolute activity scale, site-specifically immobilized L91pPa lysozyme was more than twice as active as unimmobilized standard lysozyme after urea treatment and was 73% more active than the nonspecifically immobilized standard lysozyme (Figure 5-5).

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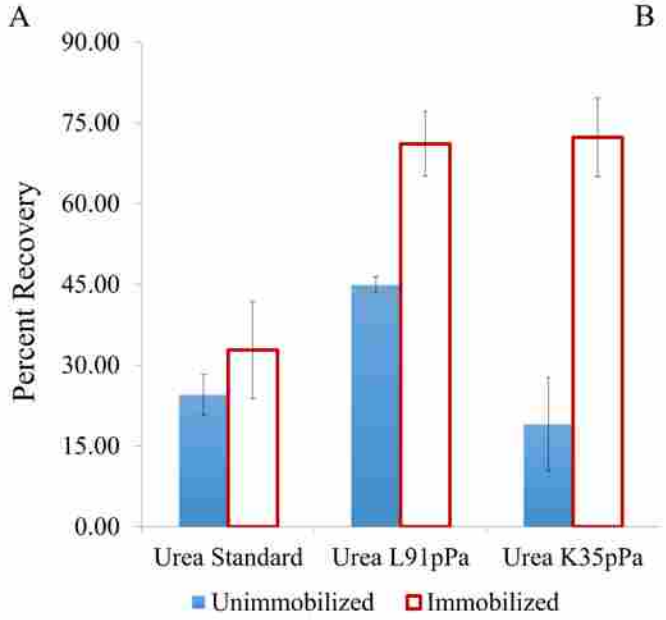

B

Type of Lysozyme	Urea Treated	Immobilized	Activity as a % of Standard	Standard Error
Standard			100	N/A
	X		24.5	3.8
	X	X	32.8	9.0
L91pPa			79.8	2.5
	X		35.8	1.5
	X	X	56.7	6.0
K35pPa			28.9	6.2
	X		5.5	8.7
	X	X	20.9	7.3

Figure 5-4: Post-Urea Incubation Stability. Each lysozyme was incubated in 2M urea at 37°C for 30 minutes and then assayed for activity. A. Percent of lysozyme activity retained post-urea incubation. B. Normalized lysozyme activity standardized to the untreated and unimmobilized standard lysozyme. (Wu et al., 2015).

5.5 Conclusion

Here we employ our recently developed PRECISE system to demonstrate for the first time site-specific covalent immobilization of enzymes at residues throughout the enzyme. Different from prior immobilization techniques, as demonstrated here, the PRECISE system is an entirely covalent immobilization that requires only a single substitution or insertion to facilitate immobilization orientation control (Ewers et al., 2005; Kalia et al., 2007; Lim et al., 2014; Seo et al., 2011; Smith et al., 2005). We have demonstrated that by controlling the immobilization orientation, greater enzyme stability can be realized than nonspecific covalent immobilization, even by 50% and 73%. Improved stability is especially important for developing enzyme biocatalysts capable of long-term reuse and thus enabling economically competitive biocatalyst use in lower margin chemical applications. As a potential industrially relevant carrier,

superparamagnetic beads were employed as the immobilized enzyme carriers. These beads provide facile recovery and reuse of the enzymes as was demonstrated during the stability assays.

We have also provided preliminary data suggesting that current simulation capabilities may be useful in selecting a good location for enzyme immobilization, with the simulation-predicted L91 site being the most stable location for enzyme immobilization under two different denaturing conditions. Overall, the PRECISE system enables a truly covalent attachment of the enzyme at potential any accessible residue and is an attractive technology to determine the optimal orientation for enzyme immobilization for biocatalysis applications.

6. Conclusions and Future Work

6.1 Thermodynamic Stability of Immobilized Enzymes: Explanations for Increased Stability

Our experimental findings on lysozyme stability support prior molecular modeling predictions that enzyme immobilization at residue 91 would increase stability over unimmobilized enzyme and enzyme immobilized at other sites (Wei and Knotts, 2011b). However, our studies also supported prior research that demonstrated that enzyme immobilization generally increases enzyme stability (Friedel et al., 2007). Dill et al have theorized that the entropic cost of folding on a surface is less than or equal to the cost of folding in solution in all instances, thus increasing the thermodynamic stability of enzymes when immobilized on a surface (Dill and Alonso, 1988; Zhou and Dill, 2001). Other molecular modelers have tested this hypothesis and found strong supportive evidence of this theory, but noted that the strength of the stability improvements rely on the specific immobilization site (Knotts Iv et al., 2008). Based on the additional stability improvements that our site-specific immobilization technique demonstrated over the traditional, nonspecific covalent techniques, we find support for this hypothesis – that the decrease in entropic cost for folding stabilizes immobilized enzymes, but that careful site selection is crucial for maximizing stability benefits.

6.2 Future Work

Given the importance of site selection for maximizing enzyme stability benefits and due to the location-specific impact of non-canonical amino acid incorporation, better techniques for evaluating the best locations for non-canonical amino acid incorporation must be developed. For proteins and enzymes that have a solved crystal structure, the choice of the most advantageous incorporation sites is simplified. The traditional rules of selecting an external, non-structurally significant lengthy loop region far from the active site or the closest approximating site might

provide a good initial region to begin looking for a viable immobilization site. In the alternative, molecular modeling may also provide an effective strategy for discovering the most stable immobilization site.

However, for the vast number of proteins and enzymes that do not have a solved crystal structure or close homology to a solved structure, molecular modeling may be quite difficult. To provide an example, the I-Tasser homology/folding-based protein structural prediction program by the Zhang Research Group at Michigan and the Quark ab initio protein structural prediction program by the same research group currently provide the most accurate protein structural prediction software as assessed by the Critical Assessment of protein Structural Prediction (CASP), the foremost competition for protein structural prediction (Roy et al., 2010; Xu et al., 2011). Despite being the most advanced and accurate protein structural prediction software, both

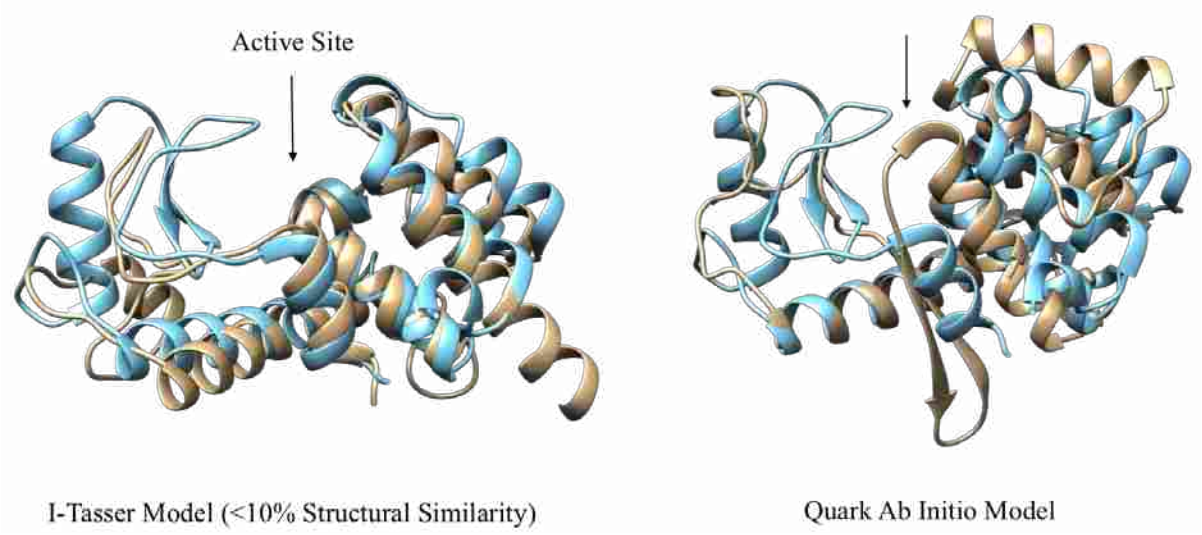

Figure 6-1: Protein structural prediction models. Models produced from I-Tasser and Quark when given the amino acid sequence of the T4 Lysozyme (PDB ID: 7LZM). The correct structure is colored in blue, the predicted structure is colored in gold.

software programs suffer from significant prediction inaccuracies where a protein lacks greater than 10% homology with a protein whose crystal structure has been previously solved (Figure 6-1).

We are currently working on a high-throughput system for testing large numbers of non-canonical amino acid incorporation sites concurrently using linear expression templates produced in the cell-free protein synthesis system that enable same-day design, protein testing, and protein production that will hopefully, work to address the difficulty of finding the most advantageous incorporation sites.

In conclusion, using the technologies of cell-free synthetic biology, non-canonical amino acids, and click conjugation, we have demonstrated a powerful new technology that enables significant activity and stability benefits in proteins and enzymes, a better understanding of enzyme-surface interactions, and that may help to simplify protein production of proteins with unique new qualities.

7. Appendix 1: Supplementary Material for Chapter 2

Enhanced protein stability through minimally-invasive, direct, covalent and site-specific immobilization

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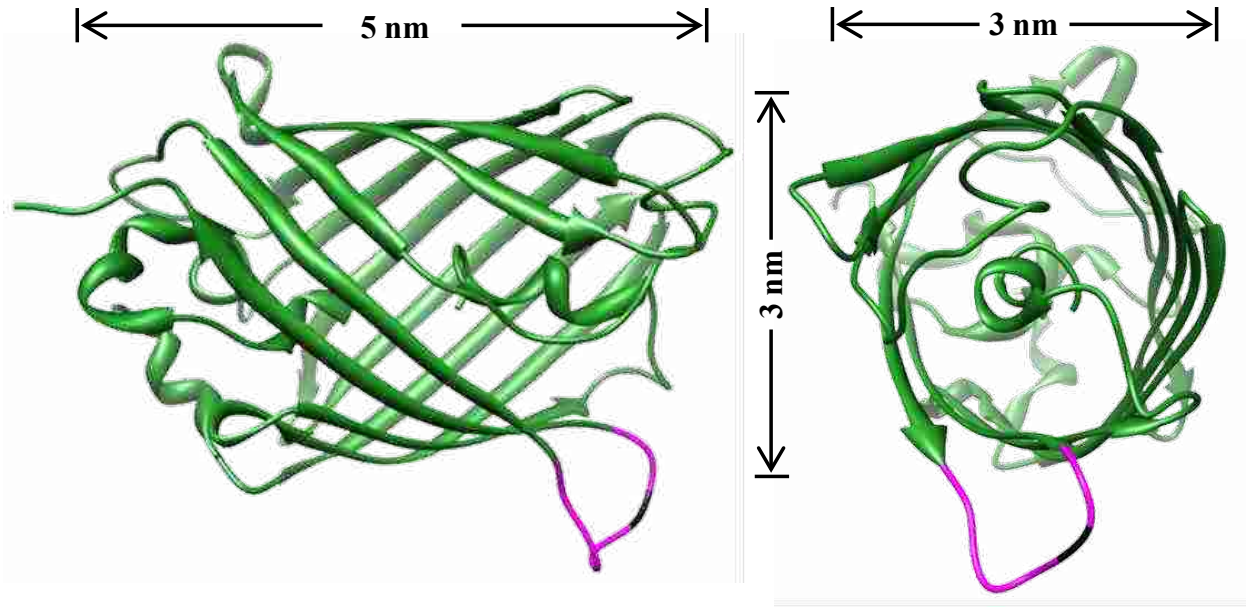

Figure 7-1: Crystal Structure of sfGFP-related Protein. The potential implications of the residue location for unnatural amino acid incorporation were investigated using the known crystal structure of a superfolder green fluorescent protein variant closely related to the pPaGFP (pdbID 2B3P).(Pedelacq et al., 2006) In the Fig. S1, the beta-barrel (dark green) is approximated as a cylinder with the loop of interest (pink) extending from the main body. The pink loop contains the location for the unnatural amino acid incorporation (black). (Smith et al., 2013).

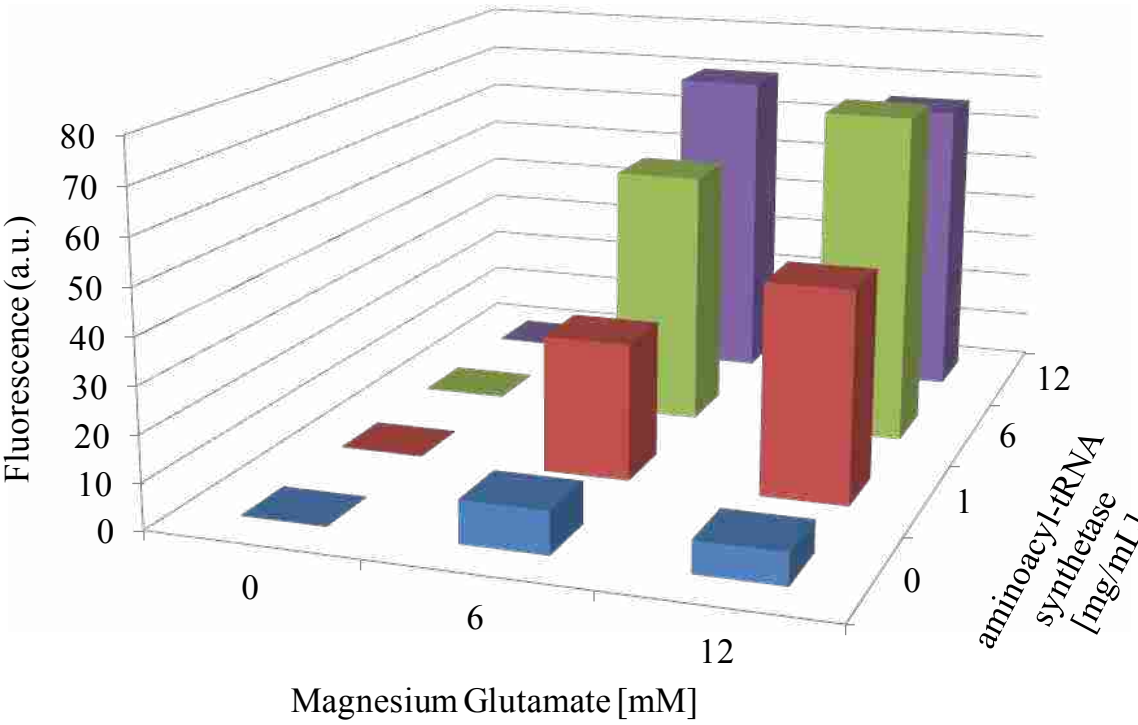

Figure 7-2: Aminoacyl-tRNA Synthetase Optimization. Cell-free protein synthesis allows for direct access to the synthesis environment, enabling optimization and maximization of synthesis cofactors. To maximize the unnatural amino acid incorporation, the effect of different concentrations of aminoacyl-tRNA synthetase and magnesium glutamate on the production of active pPaGFP was assessed. (Smith et al., 2013).

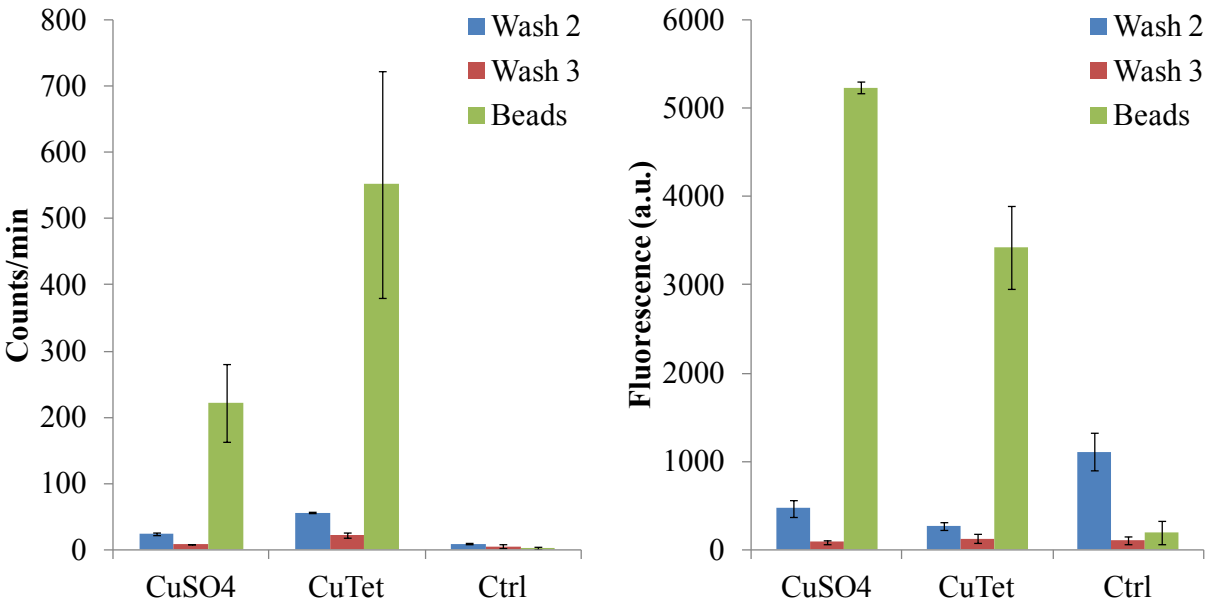

Figure 7-3: Scintillation and Fluorescence Data for Washes after pPaGFP Immobilization. To ensure that the proteins were not remaining in solution or non-specifically binding to the magnetic beads, the beads were washed 3 times using a PBS-Tween buffer. Each wash consisted of isolating the magnetic beads, removing the supernatant, and resuspending the beads in 100 µL PBS-Tween buffer. The final suspension (labeled "Beads") was in 100 µL PBS and was vortexed directly prior to analysis for a uniform suspension of beads. Displayed above are the results from liquid scintillation and fluorescence analysis of supernantant from washes 2 and 3, along with the final suspension containing beads. During the washing process, the unclicked pPaGFP was removed to background or statistically insignificant levels. For the control reactions containing no copper, the resuspended beads contained no or insignificant levels of pPaGFP. The prewash and wash 1 results were removed to provide appropriate scaling. Error bars = standard deviation, n=2. (Smith et al., 2013).

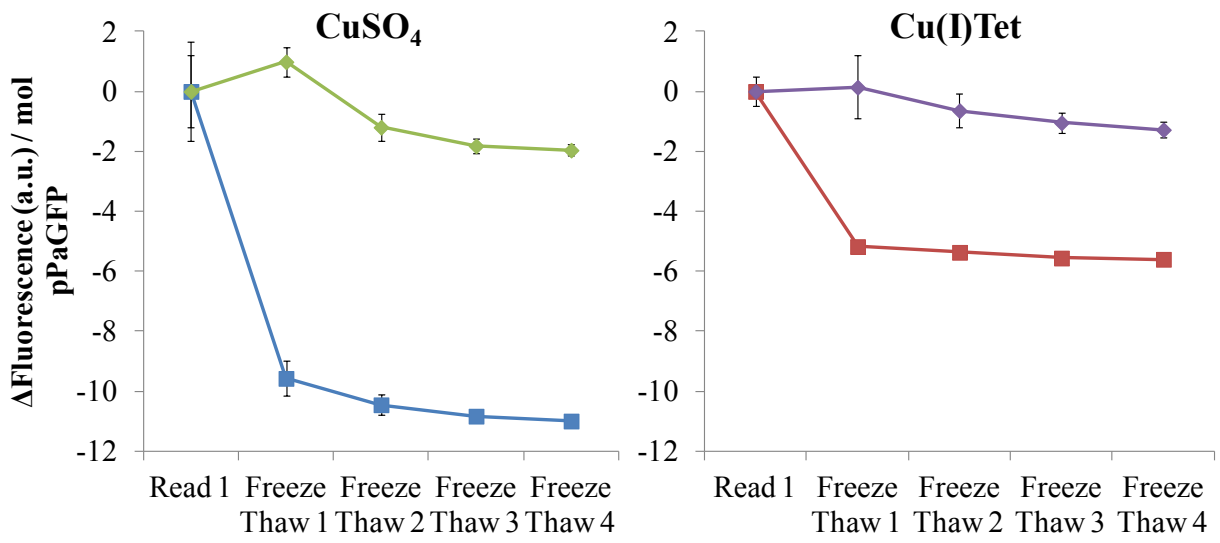

Figure 7-4: Freeze-thaw Activity for Free pPaGFP in Copper-containing Solutions. Free pPaGFP was incubated under the same conditions as bead-immobilized pPaGFP and then was left in its respective reaction solution. The unbound pPaGFP was subjected to multiple freeze-thaw cycles (squares) or incubated at 4 ºC (diamonds) and assayed for activity. Each freeze-thaw cycle consisted of an incubation for 20 min at -80 ºC followed by a 20 min incubation at room temperature. Samples left unfrozen were maintained at 4 ºC for 40 min between assays. The results suggest that incubation with copper plays little role in terms of stability during freeze-thaw cycles. Error bars = standard deviation, n=2. (Smith et al., 2013).

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