PROTEIN ENGINEERING WITH COMPUTATIONAL MODELING

DISSERTATION

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by

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Vita

Carlo Yuvienco was born in New York, New York on March 22nd, 1983 to Dr. Francisco P. Yuvienco and Elizabeth T. Yuvienco. His academic and professional pursuits would reside in New York until the time of this work, starting with his secondary education at Stuyvesant High School. This was followed by an undergraduate education in chemical engineering at the Cooper Union for the Advancement of Science and Art, culminating in a Bachelor of Engineering (granted in 2005). Carlo then was employed at Pall Corporation (East Hills, NY) as a research scientist, working on the development of leukocyte-reduction filtration media. After two years of industry experience, Carlo pursued a Master of Science in biomedical engineering at the Polytechnic Institute of New York University (granted in 2010), immediately preceding the pursuit of his doctorate in biomedical engineering under the advisement of Prof. Jin Kim Montclare, Ph.D. at the now New York University - Polytechnic School of Engineering, in the Department of Chemical and Biomolecular Engineering. From 2008 to 2014, Carlo worked on several projects in the field of protein engineering under the guidance of Prof. Montclare, having conducted experiments in various labs within New York University, the Navy Research Laboratory, the Wright-Patterson Air Force Base Materials and Manufacturing Directorate, and the City University of New York. During the course of his doctoral research, he and his research projects have been supported by the National Science Foundation (GK-12 Program, DGE-0741714; NYU MRSEC Center, DMR-0820341; I-Corps Program, IIP-1332165; DMR-1205384), the Air Force Office of Scientific Research (FA-9550-07-1-0060 and FA-9550-08-1-0266), and the Army Research Office (W911NP-10-1-0228).

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ABSTRACT

PROTEIN ENGINEERING WITH COMPUTATIONAL MODELING

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With the advancement of technologies to probe and manipulate biophysical matter, the scientific community continues to ever better engineer biological systems with the complexity and elegance in design that is necessary to address biomedical challenges. The growing maturity of the field of protein engineering is a testament to this proclamation. Herein, two fundamental ideas are explored. In Chapter I, an evaluation is presented on the effects of the incorporation of a non-canonical, fluorinated amino acid into a protein-based block copolymer. The ramifications of these results, and similar others in the field, on the promise for predictable tuning of the physicochemical behavior and properties of protein-based materials are emphasized. In Chapter II, an alternative application of an endogenous protein is examined, harnessing its inherent form and function. Hypotheses postulate the ability of a coiled-coil protein, of particularly high oligomeric order, to facilitate the delivery of small molecule therapeutics for the treatment of osteoarthritis, whilst addressing dominant hurdles pertaining to drug localization. This complete body of work rests on the themes of control and repurposed application of biophysical matter, contributing to the formalization of engineered systems within protein science.

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Chapter 1

Improved Stability and Half-Life of Fluorinated Phosphotriesterase Using Rosetta

1.1 Introduction

1.1.1 Rosetta and Protein Engineering

Computational tools are widely used for protein engineering [ref]. Rosetta suite was first developed in University of Washington [ref]. Baker et al. adapted this suite for prediction of three dimensional structure of proteins. This suite provides a handful of protocols for analyzing and mutating protein structures. The simulation replies heavily on knowledge-based potentials. It is a suite of libraries and tools for macromolecular ligand docking, to thermo-stabilize proteins, to design a hydrogen-bond network, to design novel protein folds, to create novel protein interfaces, and to design enzymes, including some containing unnatural amino acids in the active sites.

1.1.2 Phosphotriesterase

PTE is a homodimeric protein composed of two monomers, each of which contains a metallo-active site.Phosphotriesterase (PTE) are enzymes, which hydrolyze organophosphates (OPs) as well as synthetic esters.[1] OPs are a synthetic class of small molecule that irreversibly inactivate acetylcholinesterase (AChE), disrupting neural transmission. AChE is an enzyme that degrades the neurotransmitter, acetylcholine, at the neuromuscular junction in the cholinergic nervous system. After the acetylcholine is hydrolyzed, the synaptic transmission would be terminated. Inhibition of AChE lead to hyper-stimulation from toxic accumulation of acetylcholine.[2] Army also adapted this protein for chemical weapons neutralization. [3]

1.1.3 Incorporation of non-natural amino acids

Several methods have been developed for the incorporation of unnatural amino acids into proteins: solid-phase synthesis (SPPS)[4], in vivo and in vitro site-specific incorporation, 16 and residue-specific incorporation (Fig. 1)1d, 17. In SPPS, activated amino acids are immobilized on a solid support and synthesized step-by-step in the reactant solution. This method is convenient for the introduction of functional groups into peptides, but it is still restricted to the yield and the expense of peptides. For example, if each coupling step has 99% yield, a 26-amino acid peptide would be synthesized in 77% final yield. To synthesis longer chain peptides and proteins bearing UAAs, biosynthetic methods have been developed. There exists two contemporary methods to biosynthetically incorporate non-natural amino acids into proteins: site-specific incorporation and residue-specific incorporation. Schultz and their coworkers18 have developed a general approach for the in vitro synthesis of proteins. The approach relies on the suppression of an amber termination codon (UAG) in the mRNA by an amber suppressor tRNA charged with the amino acid analog. This

method has been well studied and developed in research of protein structures and functions19.

Methods to incorporate amino acid analogues site-specifically into proteins in vivo greatly expand research of unnatural amino acids. We are not only able to synthesize large amounts of protein, but capable of overcoming potential problems including post translational modifications. An in vivo site-specific method to incorporation UAAs was developed by Schultz and coworkers21. A stop codon at the position of interest is encoded in the mRNA. For in vivo site-specific UAA incorporation, an orthogonal aminoacyl-tRNA synthetase charges an orthogonal tRNA with particular UAA, and the suppressor tRNA would help the incorporation of UAA with recognition of a stop codon. As cells contain 20 aminoacyl-tRNA synthetase/suppressor tRNA pairs, a new one is required for the incorporation. An orthogonal aminoacyl-tRNA synthetase/suppressor tRNA pair based on a TyrRS/tRNATyr pair in the *Methanococcus jannaschii* has been engineered for use in *E. coli* for the incorporation of tyrosine analogs21a.

As an alternative to site-specific incorporation, residue-specific incorporation has been developed in which a natural amino acid is replaced with an UAA. Auxotrophic strains or organisms that cannot biosynthesize a particular natural amino acid, has been used to introduce multiple UAAs throughout the protein sequence. UAAs that are isosteric to natural amino acids are capable of being recognized by the natural aminoacyl-tRNA synthetase (aaRS), charging the appropriate tRNA enabling the introduction of UAA into the protein sequence without alteration of the biosynthetic machinery. However, to introduce UAAs with gross differences from the natural amino acids, further engineering of the aaRS is required. To incorporate refractory methionine analogs, Tirrell and coworkers engineered additional copied of the methionyl-tRNA synthetase (MetRS) by adding the MetRS gene under constitutive promotor22. Alternatively, Schimmel and coworkers mutated editing pocket of valyl-

tRNA synthetase (ValRS) to facilitate the incorporation of analogs that normally would not be accepted by endogenous aaRS23. Finally, Kast and coworkers generated a mutated phenylalanyl-tRNA synthetase (PheRS), ePheRS* under a constitutive promoter, with a large binding pocket (T251G) and showed relaxed specificity.24

1.1.4 Fluorinated amino acids in proteins

Fluorinated amino acids (FAAs), represent a unique class of UAAs. They have different bond energies, electron distributions, and hydrophobzgicity 26 as compared to their hydrogenated counterparts. As we compare the structure of fluorocarbon groups, t(Bh)e C-F bond is highly dipolar while the hydrocarbon is less. The C-F bond is roughly 0.24 longer than C-H bond26 (Table 1). While in some cases the global replacement of hydrophobic amino acids with fluorinated analogs has led to the stabilization of protein structure27, it has all been shown that in some cases they can reduce the thermodynamic stability28.

1.1.5 Scope of work

The primary goals of this work were to done adapt Rosetta for phosphotriesterase. Overall, with incorporation of *p*FF into protein, we will be able evaluate the performance of scoring function. In advance, we would evaluate the shelf life and thermostability of phosphotriesterase.

1.2 Methods

1.2.1 General

All chemicals, reagents, and substrate were purchased from Sigma. T4 DNA ligase was purchased from Roche. DNA sequence was confirmed by Eurofins MWG Operon.

96-well plates were purchased from Thermo Fisher Scientific (Waltham, MA).

1.2.2 Recombinant gene construction

pQE30-S5 was used as described before. The pQE30-104A plasmid was prepared with forward primers (5-GATGTGTCGACTGCCGATATCGGTCG-3, Fisher Scientific), reverse primers (5-CGACCGATATCGGCAGTCGACACA-3, Fisher Scientific). The PCR parameters were set as follow for 18 cycles: initial denaturation in 95 °C¡++¿ for 30 seconds, sequential denaturation in 95 °C¡++¿ for 30 seconds, annealing in 55 °C¡++¿ for 1 minute, and extension in 68 °C¡++¿ for 4 minutes. The mixture was then incubated 37 °C¡++¿ C overnight with DpnI to digest methylated parent DNA strands, which lack the desired mutation. DNA sequence was further confirmed by Eurofins MWG Operon.

1.2.3 Protein Expression

Mutant and wild type plasmids were transformed into E. coli phenylalanine auxotrophic strains (AF-IQ cells).[5] Electroporation was done at 25 F, 100 Ohms, 2.5 kV (Biorad Gene Pulser II). Cells were plated on agar plates containing 200 g/mL ampicillin, 34 g/mL chloramphenicol. A Single colony was picked and grown in medium (M9 medium supplemented with 0.2 wt % glucose, 35 mg/L thiamine, 1mM $MgSO_4$, 0.1mM $CaCl_2$, 200 g/mL ampicillin, and 34 g/mL chloramphenicol) with 20 mg/L of 20 amino acids at 37 C, 300 r.p.m. Afterwards, 250 mL of M9 medium for large-scale expression was innoculated 1:50 with an overnight culture. After optical density reached 1.0 at 600 nm, media shift was carried out by washing the cells three times with 0.9% 4 °C¡++¿ NaCl. Cells were then transferred to M9 minimal medium containing either 20 amino acids or 19 amino acids (-Phe). pFF-PTE and pFF-104A expression media were supplemented with and 3 mM of pFF and 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) to induce protein expression. 1mM of $CoCl_2$

was added in each post-induction medium. After three hours incubation at 37 C, 300 r.p.m., the cells were harvested and then resuspended with 20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, 10% glycerol (pH 8.0) and 1 µmol $\cdot$ L^{-1}¡++¿ $CoCl_2$. Cell lysate was sonicated on ice for 1.5 minutes and then a clarification spin was performed (20, 000 g, 4 °C¡++¿, 30 min). Clarified supernatants were loaded into a His Trap column (G.E Healthcare, Piscataway, NJ) using KTA FPLC purifier (G.E. Healthcare, Piscataway, NJ). Protein elution was generated using elution buffer B (20 mM Tris-HCl, 500 mM sodium chloride, 500 mM imidazole (pH 8.0)). The purified samples were then transferred for buffer exchange using 3X 4L 20 mM phosphate buffer (pH 8.0). Dialyzed protein was subjected to kinetic assays immediately.

1.2.4 Thermo-stability and Stability of Phosphotriesterase

Here, we introduce the thermo-stability of phosphotriesterase. *p*FF-ECE, respectively.

1.2.5 Enzyme Kinetics

wild-type counterparts. Moreover, fluorination yielded robust elastic network formation for all three protein polymers at elevated temperatures.Kinetics will be done in 200

1.3 Results

This is short description of my results.

I am testing this thing out.

1.3.1 Biosynthesis of phosphotriesterase

I should attach one photo of my gel here.

1.4 Discussion

While the secondary structure appears conserved with respect to diblock variants, the supramolecular behavior appears to have been altered, suggesting

1.4.1 Protein design

In the realm of synthetic chemistry, there has been a long standing interest in the physicochemical properties of fluorinated polymers. Self-assembly into higher-order structures has gained particular focus, in the cases of semifluorinated dendritic Janus particles and fluorinated amphiphiles, which affect assemblies on the supramolecular scale in different ways, and despite the early successes in the incorporation of fluorinated amino acids into protein polymers, little has been accomplished in the field with respect to material characterization. Our studies demonstrate that fluorinating biopolymers cannot only impact the secondary structure and T_t, but, more importantly, influence the supramolecular assemblies and mechanical properties. While these fluorinated protein polymers exist as soft gels, the observed modifications to the self-assembly and rheological properties from the incorporation of non-natural amino acids provides a precedence and an opportunity for tuning protein-based materials. This provides a novel and alternative route for tuning smart materials that rely on gel mechanics, in the case of applications in tissue engineering, and thermoresponsive transition, in the case of applications in drug delivery.

1.4.2 Future work

Further optimization devoted to the incorporation efficiencies of fluorinated amino acids into these block copolymers is necessary for further practical development of the compositions. More uniform incorporation will further promote consistency in the

observable physicochemical properties of the block copolymers, notwithstanding any inherent stochastic behavior that embodies the self-assembly processes. This may be carried out by either carrying out the biosynthetic expression under stricter selective control, or by engineering the expression system to one more based on orthogonal transcription technologies.

Beyond the improvement in incorporation methods, additional attention should be devoted to the functional characteristics of COMP, as it exists as part of the block copolymer. These observations on COMP - specifically its ability to 1) oligomerize and 2) bind to small molecules - should be carried out at conditions preceding and following thermo-actuated transitions, as they are measured in the body of this work. This work may seek, for example, to correlate the thermal transition of the elastin domain to the incrementation of COMP oligomerization states, providing insight into the engineered application of elastin-like peptides as oligomerization chaperones. Assessment of binding properties of the COMP domain, and/or the other elements of the block copolymers, will further promote these inventions toward *in vitro* and *in vivo* applications; the challenge lies with adopting viable small molecule as payload candidates.

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