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Biochemical Study of Engineered Fluorescent Proteins as Calcium Sensors and the Effect of Calcium and PH in Cell Reproduction and Protein Expression

Malcom Arturo Delgado
Georgia State University

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BIOCHEMICAL STUDY OF ENGINEERED FLUORESCENT PROTEINS AS CALCIUM SENSORS AND THE EFFECT OF CALCIUM AND PH IN CELL REPRODUCTION AND PROTEIN EXPRESSION

Presented in Partial Fulfillment of Requirements for the Masters Degree in the College of Arts and Sciences Georgia State University

2009

By

MALCOM ARTURO DELGADO

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December 10, 2009
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By

Malcom Arturo Delgado

Under the Direction of Dr. Jenny J. Yang

ABSTRACT

Calcium plays important roles in both eukaryotic and prokaryotic cells. Its actions help to stabilize cell synthesis, growth and development.

In this thesis, studies have been completed to determine effects of calcium and pH on bacterial cell growth and protein expression using the bacterial cell strain *E.coli* BL21(DE3). Our studies demonstrated the addition of calcium addition in the media does not affect growth but increases protein expression, while reducing the pH from 7 to 4 through the addition of 10mM EGTA in LB media inhibits both.
Additionally, we report studies on the design, expression, and purification of fluorescent mCherry variants and their differences in their optical properties, including: extinction coefficients, quantum yields and pKa values. Also, we report progress in the crystallization of two GFP calcium sensors: G1 and D1, using 13 and 15% PEG 4000 and 3350 respectively in 50mM HEPES buffer (pH 6.8-7.0) in an effort to optimize crystallization.

INDEX WORDS: Calcium, Protein mCherry, Green Fluorescent Protein (GFP), E. coli. X-ray crystallography, Protein expression, Fluorescent proteins, BL21(DE3).
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Office of Graduate Studies
College of Arts and Sciences
Georgia State University
December 2009
DEDICATION:

This thesis is dedicated to my father and God lord, because everything I do, He always makes it possible. To my teachers and professors, class and lab partners, and to those whose support and advice have helped me along the path to success. To my family, of course, that has given me the support, the help, courage, self-awareness, education, spiritual values etc. that have been a key in my life for my dedication, to achieve my goals, and be a better person. Finally to myself since my wise decisions, efforts and perseverance have made possible the accomplishment of this and my future career.

Esta tesis esta dedicada a mi padre y senor Dios, por que todo lo que hago, El siempre lo hace posible. A todos mis profesores, compañeros de clase y laborartorio y todos aquellos que con ayuda y consejos me ayudaron atravez de este camino hacia el exito. A mi familia, porsupuesto, quienes tambien me han dado el soporte, la ayuda, coraje, ejemplo, educacion, valores espirituales y mas que han sido la llave en mi vida de dedicación, para conseguir mis metas, y ser mejor persona. Finalmente a mi mismo ya que mis decisiones, esfuerzos, y perseverancia han hecho posible el logro de este trinfo.

“Surprising the act of nature:
create Chemistry
for the creation of science.”

m.e.c
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CHAPTER I:

1. BACKGROUND OF CALCIUM AND CALCIUM SENSORS FOR SIGNALLING IN LIVING CELLS

1.1. Why calcium is so important in intracellular processes?

Calcium is a soft gray alkaline metal that has the atomic number 20 and atomic mass of 40.078 g/mol. This mineral is the most abundant metal in our bodies and a high consumption of calcium is needed not only for building strong bones, but also for many other functions including: conducting nerve impulses, contracting muscles, secreting hormones, aiding blood clotting and activating enzymes [1]. Cells are the basic compositions of the human body and to maintain their functions, they uptake calcium to activate different protein signaling pathways [2] (Figure 1.1). Intracellular processes contribute directly or indirectly to different levels of cellular functions, ranging from genome sequence to transcriptional and translational profiles [3].

Normally, during rest conditions, the intracellular calcium concentration is ~0.1 to 1.0µM [4] (Figure 1.2). Diseases directly related to overloading and/or depleting the intracellular calcium conditions includes osteoporosis (low calcium balance) and hypocalcaemia (High calcium concentration in the blood) [1, 5]. On the other hand, acute or chronic pancreatic diseases such as cardiovascular diseases, neurodegenerative diseases and cancers can be attributed to the malfunction of intracellular Ca^{2+} signaling processes [6]. Advances in genomics or proteomics technology have provided the tools to better understand of intracellular processes and corresponding cellular functions [7] [8], direct, novel and effective methods to real-time monitor calcium-dependent protein-protein interactions in living cells are still needed with great desire.

Many different proteins are sensitive to calcium concentrations inside (and outside) cells. It is important to study calcium binding proteins and probe cellular calcium responses so that we can understand molecular basis. Related diseases can be explored in molecular mechanisms by
monitoring the intracellular activities of the proteins which also provide important targets for the treatment of diseases.

Figure 1.1: Cell pathways that attribute the presence of calcium for different intra and extracellular functions. It plays important roles in cell development. It is a key determinant for many intracellular pathways at different cell locations for both eukaryotic and prokaryotic cells.

Figure 1.2: Predictable intra and extra cellular calcium concentrations in both eukaryotic and prokaryotic cells. Different organelles in eukaryotic cell possess different concentrations of calcium that have been unknown for other studies.

1.2. **Calmodulin (CaM): A calcium binding protein**

Calmodulin modulated protein (CaM) is a protein expressed in the cytosol of all eukaryotic cells whose function is dependent upon calcium binding (Figure 1.3). The main function of CaM is to regulate different cellular processes by interacting different membrane receptors and channels. Some of these regulating pathways include the mediation of inflammation, metabolism, apoptosis, muscle contraction, intracellular movement, short-term and long-term memory, nerve growth and the immune response[9]. CaM is expressed in different types of cells at different sub-cellular locations including the cytoplasm, within cell organelles, and sometimes within the plasma, and other organelle cell membranes. CaM has been highly conserved through evolution.
and has been determined that Ca$^{2+}$ plays a crucial role in the metabolism and physiology of eukaryotes. CaM protein serves as a calcium sensor and transducer of calcium signals in the cell.

CaM is an essential calcium-binding protein that binds to and activates a diverse population of downstream targets (calmodulin-binding proteins: CaMBPs) that carry out its critical signaling functions (Figure 1.4). CaM-BP’s are diverse group of targets, whose interactions with CaM can be subdivided into calcium-dependent and calcium-independent modes of binding and regulation. Ca$^{2+}$-CaM complex can bind to target proteins to alter their function, acting as part of a calcium signal transduction pathway. Ca$^{2+}$-CaM binding proteins include kinases, phosphatases, second messenger signalling proteins, cytoskeletal and muscle proteins[9]. These proteins can be either Ca$^{2+}$-activated or Ca$^{2+}$-inhibited. Ca$^{2+}$-CaM can bind to target proteins resulting in: neurotransmitter release, muscle contraction, metabolism and the inflammatory response.

There is no general model of target recognition and activation by CaM; instead several mechanisms of interaction have been described for different targets. CaM is thought to activate myosin light chain kinase (MLCK) and CaM kinase II (CaMKII) by displacement of their auto-inhibitory domains. With anthrax adenylate cyclase exotoxin activation, CaM was found to insert itself between two domains of the exotoxin. Other mechanisms of activation include binding of the CaM C-terminal alone, binding of CaM in an extended conformation, and dimer formation with its target. This mechanistic diversity means that there is no conserved amino acid sequence for CaM binding, however several different binding motifs have been recognised based on the positioning of conserved hydrophobic residues. CaM binding proteins can have one or more CaM-binding domains.
CaM undergoes a conformational change upon binding for calcium ions, which enables it to bind to specific proteins for a specific response. CaM can also undergo post-translational modifications, such as phosphorylation, acetylation, methylation and proteolytic cleavage, each of which can potentially modulate its actions[10]. Calcium-free apocalmodulin (ApoCaM) is structurally different from Ca\(^{2+}\)-CaM and utilizes unique binding motifs, which show greater homology than Ca\(^{2+}\)-CaM binding motifs. ApoCaM can bind to other target proteins, eliciting different cellular responses. Some of these include neuroproteins, receptors, second messenger signalling proteins, cytoskeletal and muscle proteins.

There are also many other regulatory proteins that bind calcium, which together form an intricate network of feedback loops to control the location, amount and effect of calcium influx.

Figure 1.3: Calmodulin is a small dumbbell-shaped protein composed of two globular domains connected by a flexible linker. Each domain binds two calcium ions. PDB entry 3cln, shown here, has all four sites filled with calcium ions and the linker has formed a long alpha helix connecting the domains.
In the late 1960's, before the discovery of calmodulin, troponin C (see, for instance, PDB entry 1tcf) was the first protein shown to be sensitive to calcium. Troponin C senses rising calcium levels and triggers muscle contraction. The structures of troponin C and calmodulin are remarkably similar, the major difference being the length of the linker connecting the two calcium-binding globular domains. The calcium-binding region or EF-hand motif of CaM is almost identical. This EF-hand motif has since been found in dozens of other calcium-sensitive proteins.

1.3. Calsequestrin (CaSQ)

Calsequestrin or CaSQ is a 400 aminoacid long peptide that binds to calcium (Figure 1.5). Calsequestrin consists of three helical domains which work together to maintain a calcium storage in the sarcoplasmic reticulum of cells after a muscle contraction. This protein effectively binds 40-50 Ca$^{2+}$ ions per protein molecule. A study in 2007 showed that a mutation (D307H) in
the protein sequence of CASQ expressed in the mouse heart was shown to relate to abnormal calcium distribution and binding capacity and resulted in a complex ventricular arrhythmia similar to Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) developed in humans[11]. The over-expression of the protein CaSQ during embryonic development was proposed to be a potent cause of premature death. Scientists think that the function of these two proteins impairs glucocorticoid action.

Figure 1.5: Three repeating domains of CaSQ protein (monomer). PDB:2VAF

Because the protein CaSQ have been considered a potential calcium binding protein due to the fact it contains 45 calcium binding sites and also because of its high availability in different cells such as mammal and plant cells, in our lab we use calsequestrin as a model protein to study key determinants for its multiple calcium binding and protein stability.
1.4. **Fluorescent proteins**

The two fluorescent proteins that will be described throughout this thesis are the Green Fluorescent Protein (GFP) and mCherry fluorescent protein. GFP comes from the jelly fish *Aequorea Victoria* and it was isolated by Dr. Osamu Shimomura. Dr. Martin Chalfie was the person to give significance to GFP as a genetic tag for various Biological phenomena; he was the first in expressing the protein in the transparent roundworm *Caenorhabditis elegans*. Dr. Roger Tsien contributed to our general understanding of how GFP fluoresces. He also extended the color palette beyond green allowing researchers to give various proteins and cells different colors. This enabled scientists to follow several different Biological processes at the same time. mCherry comes from different mutations of the tetramer DsRed fluorescent protein that comes from the coral *Discosoma sp.* The architecture of GFP and mCherry proteins are similar based on their shape and chromophore formation (Figure 1.6).
1.4.1. Chromophore formation of fluorescent proteins

The chromophore of GFP was first found to form through the cyclization of the tripeptide Ser65-Tyr66-Gly67[12], located inside of the β-barrel composed of 11 anti-parallel strands and a single central α-helix (Figure 1.7 and Figure 1.7a-c). The chromophore of mCherry was found to form through the cyclization of Gln66-Tyr67-Gly68. The residue Gln adds an extra double bond to the conjugated system of the chromophore making it to fluoresce at a higher wavelength.
Figure 1.6. Chromophore comparison for GFP and mCherry proteins. The biggest difference between green fluorescent protein and its red analog, DsRed, is that the chromophore of DsRed has an extra double bond (drawn in yellow) which extends the chromophores conjugation and causes the red-shift.

Short helices cap the ends of the barrel [13-14] [13]. The active chromophores of these fluorescent proteins are tripeptides and require the presence of oxygen to mature [15]. The cylinder of GFP has a diameter of about 30 Å and a length of about 40 Å [14]. Although the chromophore is located on the central helix within several Å’s of the cylinder center, both a polar and polar amino acid side chains such as S202, T203, I167 or G148 and immobilized water molecules surround the chromophore.

Shimomura analyzed the denatured GFP by proteolysis [12]. He deduced the structure of the chromophore in 1979 and the result indicated that the chromophore is a 4-(hydroxybenzylidene) imidazolidin-5-one attached to the peptide backbone through the 1- and 2-positions of the ring. The structure of the chromophore was confirmed by x-ray and time-resolved fluorescence.
analysis. The tightly constructed β barrel protects well the chromophore, which contributes to the overall stability, temperature- and denaturant-resistance, and prohibition of proteases, oxygen or water molecular access. GFP and mCherry are highly resistant to denaturation and its fluorescent properties are unaffected in the presence of 6 M guanidine hydrochloride, 8 M urea or 1% SDS at 90°C; is stable over a broad pH range (5 to 12); and can tolerate various proteases such as trypsin, chymotrypsin, papain, subtilisin, thermolysin and pancreatin [16].

Physical and chemical studies of purified GFP and mCherry indicated several important characteristics relating to their specific structure. Extensive hydrogen bond interactions within the proteins frame and water molecules have significant effects on the chromophore resulting in different folding status [17]. Tsien proposed an autocatalytic biosynthetic mechanism accounting for the spontaneous chromophore formation in various GFP-expressing organisms [18], which suggested chromophore formation through autocatalytic cyclization and oxidation from residues Ser65, Tyr66, Gly67 (Figure 1.7b-c) consisting of an internal tripeptide motif that enables the protein to emit fluorescence in the absence of a cofactor [19]. Certain mutations in the chromophore affect the rate of chromophore formation in vivo and alter the spectral characteristics of the mature protein. The chromophore formation of S65T-GFP was monitored in vitro, where an ordered reaction consisting of three distinct steps was observed (Figure 1.7b-c). Protein folding occurs fairly slowly ($k_f = 2.44 \times 10^{-3} \text{ s}^{-1}$) prior to any chromophore modification. Then, an intermediate step occurs including cyclization of the tripeptide chromophore motif ($k_c = 3.8 \times 10^{-3} \text{ s}^{-1}$). The final and slower step ($k_{ox} = 1.51 \times 10^{-4} \text{ s}^{-1}$) involves oxidation of the cyclized chromophore [20].

To gain a comprehensive understanding of the reaction mechanism of GFP and mCherry chromophore biosynthesis is of fundamental scientific interest. In addition, knowledge of the
reaction pathway and identification of intermediates have significant practical and medical implications. Wachter’s group probed the crystal structure of the Y66L variant of GFP and their results supported the Cyclization-Oxidation-Dehydration mechanism for chromophore maturation [21]. To understand chromophore formation, Enoki and coworkers used pH 2.0 Tris-HCl to denature GFP and carried out research on kinetic refolding of GFP in vitro. They investigated the kinetic mechanisms of a mutant (F99S/M153T/V163A) of GFP, which is known to mature more efficiently than the wild type protein from the acid-denatured state. The kinetics of the refolding of the mutant suggests at least five kinetic phases [22]. GFP produces fluorescence when the chromophore is excited at 395nm and 488nm range and emits fluorescence at 509 nm while mCherry is excited at 520nm emitting in the 586nm.

Different mutations have resulted in various GFP variants with the characteristics of high intensity, low photo bleaching, greater stability, and low thermo sensitivity. These variants also have various excitation or emission wavelengths. The mutations around the GFP chromophore were shown to alter the optical properties of the protein. Some mutations result in the loss of the emission at 509 nm and the preservation of the excitation at 395/488 nm, such as S202F and T203I. Moreover, mutation I167T causes a reversed ratio of the sensitivity at 26 395 nm to 475 nm.

An enhanced green fluorescent protein (EGFP) was obtained through a mutation S65T in GFP. Following this mutation, EGFP exhibited improved fluorescence intensity and thermo-sensitivity relative to wild-type GFP. The shift of excitation wavelength can provide us with multiple color-fluorescent proteins, such as blue fluorescent protein (BFP), cyan fluorescent protein (CFP), green fluorescent protein (GFP), yellow fluorescent protein (YFP) and red fluorescent protein (RFP). The fluorescent protein properties of different GFP variants are
summarized in (Table 1)[23]. On the other hand, mutations F99S, M153T, and V163A that assist in folding of GFP at 37°C [24]. These mutations have little effect on the chromophore and are sites that we can utilize for the calcium binding site sequences. Fluorescent proteins (FP’s) are primarily being used for in vivo cell labeling [25] [26-27].

Proteins tagged with different GFP and mCherry variants can be visualized in cells with low light intensities over many hours and with minimal photo-bleaching. This permits intracellular protein pathways or dynamics to be analyzed in detail. The increased stability and brightness of EGFP also enables the intracellular fluorescent signal from chimeras to be correlated to standard GFP solution, permitting the quantification of molecules visualized in cells. Meanwhile, two-color fusion fluorescent protein can be used for fluorescence resonance energy transfer (FRET) to monitor intracellular interactions.

The extensive applications of GFP and mCherry are similar. They allow scientists to probe chromophore formation mechanisms, accelerate the chromophore maturation, develop various fluorescent protein variants with characteristic optical properties, and enhance the fluorescence intensity and stability of fluorescent protein variants for creating better fluorescent reporters or genetic probes capable of tracking a variety of intracellular dynamic events in a non-invasive mode is of major concern. Although GFP has attracted tremendous attention due to its potentially useful characteristics, and the chromophore structure has been confirmed, rather little is known about the detailed mechanisms of folding and chromophore formation of GFP in vitro and in vivo. The mechanisms of chromophore formation are still not well understood.
Figure 1.7a: Model structure of GFP and its secondary structure. EGFP is composed of 11 β strands and 3 helices connected through loops, which formed a compacted β-can structure. The chromophore of GFP is located in the middle of β-can. The chromophore of GFP is located in the middle of the β-can.

Figure 1.7b: Three-step mechanism of GFP chromophore formation. Pre-cyclization, intermediate and the mature chromophore
Figure 1.7c: Three-step mechanism of GFP chromophore formation. The three steps of GFP chromophore formation exhibit the formation process of a pre-cyclization structure, an intermediate structure and the mature chromophore.

1.5. **Protein probes designed to measure calcium in cells**

1.5.1. **Engineering Ca\(^{2+}\) sensors for the study of Ca\(^{2+}\) ions in specific cell locations using CaM and fluorescent proteins**

Important Ca\(^{2+}\) signals in the cytosol and organelles are often extremely localized and hard to measure. There is a great interest to develop Ca\(^{2+}\) sensors/probes to monitor Ca\(^{2+}\) concentration at different cell locations. To detect calcium signaling in living cells in real time, Tsien’s group created genetically encoded sensors for monitoring intracellular analytes “Chameleon” proteins as biological sensors based on FRET[28] (Figure 1.8).
Figure 1.8: Calmodulin (CaM) mutations can tune the Ca\textsuperscript{2+} affinities in the range of 10\textsuperscript{-8} to 10\textsuperscript{-2} M. Free Ca\textsuperscript{2+} dynamics have been visualized in the cytosol, nucleus and ER of HeLa cells. Binding of Ca\textsuperscript{2+} makes CaM wrap around the M13 domain, increasing the fluorescence resonance energy transfer (FRET) between the flanking GFP. (Rudolf et al., 2003).

Two different fluorescent protein (BFP or CFP) and (GFP or YFP) were fused with calmodulin to detect the presence of calcium in cells. Calcium ions stimulate conformational change of the complex causing the fluorescent protein to emit fluorescence at specific wavelength [28].

Although this has been an elegant technique, CaM-FRET based calcium sensors have many drawbacks. The problem faced by these calmodulin complexes occurs with the fluorescence resonance energy transfer (FRET), which relies on a distance of 1/r\textsuperscript{6} [28]. FRET-based methods require the optimal distance between the donor and the acceptor. Larger fluorescent proteins with a 4.2-nm long and 2.4-nm diameter barrel occupy much of the useful FRET distance and result in the decrease in practical maximal FRET efficiencies. In addition, free CaM, competes with the
GFP sensor, and other target proteins, bind to CaM with weaker affinity, but still competes with GFP [28].

In addition to this, the application of this class of protease sensors for in vivo imaging of protease action is limited by problems of translocation in vivo due to the typical large size of fluorescent proteins and poor fluorescence resonance energy transfer efficiency in certain environments due to poor orientation. Also, the quantitative measurement of calcium activity is partially limited by problems associated with photo bleaching [29].

1.5.2. **Approach to develop fluorescent calcium sensors**

In the look for better calcium sensors that would have a living cell affinity, many ideas have been explored. Many approaches and designs have been developed to perform this work on these host proteins, but although there have been successful results, the ending products do not satisfy the ability for analysis in the intra molecular level of cells. In our research, we have analyzed the red and green fluorescent proteins (mCherry and GFP) from *Discosoma* Red and *Aequorea* victoria to investigate and engineered potential hosts for calcium binding functions. Since these proteins form a chromophore with high absorbance and fluorescent values, they have been used to develop metal binding sites in their sequences by the insertion of commonly found metal binding motifs for an optimal biosensor design. Recent research in our laboratory with both mCherry and GFP has demonstrated that these protein constructs produce UV-absorbance emission shifts upon binding of calcium and their smaller size make them more functional for in-cell analyses.

Our lab has developed several calcium binding fluorescent proteins to study key determinants for calcium binding. Different calcium binding motifs from calmodulin (CaM) have been grafted
into the GFP at different locations. In addition, these calcium binding motifs have been grafted into the red fluorescent protein mCherry (Table 4.). Furthermore, calcium binding sites have been designed mutating both sequences of GFP and mCherry. One example is the protein mutation GFP 7E15 created by the graduate student Ada Tang, which have shown good calcium binding responses.

In the past different protein variants of EGFP were designed and tested by Dr. Jin Zou and graduate students Ada Tang and April Ellis. Different locations in the sequence were selected to mutate residues that would bind to calcium and also grafting calcium binding motifs of CaM. Similar technique was implemented for mutating and grafting the EF hand motif of CaM into mCherry. Proteins variants of GFP were expressed to compare chromophore changes in the sequence and results showed that protein fluorescent intensity for all variants were not favorable. The diagram in (Figure 1.9) shows the steps for this approach.
1.6. Protein crystallography

The crystallography method was first developed in Europe in the 18th century and the theory of geometric crystallography was not introduced until the nineteenth century. In 1895 Roentgen discovered X-rays, which have played a dominant role over the last 100 years for in material molecular structure studies. In 1820, Fraunhofer constructed the first practical diffraction gratings, which Laue later used in 1912 to demonstrate that X-ray diffraction could be used to explore the structure of crystals [30]. Protein crystallography has advanced significantly in the last decade. The results have made major contributions to the fundamental understanding of proteins and specific functions in many Biological processes. The study also has given potential insights into problems associated with protein recognitions important for drug development. Recently, improvements in computer technology have led to new methods for phase improvement, refinement of protein crystal structures and objective assessment of their likely
correctness. From the crystal unit cell, crystals are 3D ordered structures than can be observed as repetitions of identical unit cells [31]. The unit cell is made up of the smallest possible volume that when repeated, is representative of the entire crystal. The dimensions of a unit cell can be described with 3 edge lengths (a, b, c) and 3 angles (alpha, beta, and gamma). The 3D location of atoms within a unit cell can be listed as their x, y, and z Cartesian coordinates. In order for an object to be seen, its size needs to be at least half the wavelength of the observing light [31].

Since visible light has a wavelength much longer than the distance between atoms it is useless to see small molecules like proteins. In order to see molecules it is necessary to use X-rays, a form of electromagnetic radiation. The x-ray diffraction from one unit cell would not be significant.

Crystals are important because they have repeated unit cells within them. Fortunately, the repetition of unit cells within a crystal amplifies the diffraction enough to give results that computers can turn into a picture [32]. To perform x-ray crystallography, it is necessary to grow crystals with edges around 0.1-0.3 mm. Crystals are formed as the conditions in a supersaturated solution slowly change. There are three degrees of saturation in solution, and crystallographers take advantage of these when growing crystals:

1) Unsaturated: State where no crystals will form or grow.
2) Low supersaturated: State where crystals will grow but no new ones will form.
3) High supersaturated: State where crystals will both form and grow.

One theory of crystal growth is to start by getting a few crystals to grow in the highly supersaturated solution. Then the crystals are exposed to a less saturated solution so they can grow. This is done either by moving the crystals or changing the saturation of the solution.

For small molecules, growing large enough crystals is relatively simple. By taking a supersaturated solution of solution and gradually changing the conditions, crystals usually begin
to grow. If left undisturbed for a few days ideally a few large crystals may grow.

In contrast, proteins are difficult to crystallize because of their complexity, their lack of defined structure and due to other types of impurities. Crystallographers usually work with small amounts of protein to control more in detail the conditions of growth. There are a number of problems that can contribute to incorrect crystal formation. Thus because of this, it is necessary to screen ideal conditions for crystallization of desired proteins. In chapter 5, we will report our progress in obtaining crystals for designed calcium sensors of GFP. This study is one of the most important steps to obtain structure integration and to identify key factors for calcium induced fluorescence change in our developed calcium sensors.

1.7. Summary of chapters

The chapters of this thesis are summarized below and the proteins that have been studied are in table 1.1.

Chapter 2 will discussed the materials and methods used throughout this thesis. Steps to design the experiments, protein expression, purification, and quantitative analysis will be described in this chapter.

Chapter 3 will show a test done to examine the effect of calcium and pH on bacterial cell growth.

Chapter 4 will show the effect of calcium binding motifs on the red fluorescent protein mCherry. Expression, purification and chromophore optical properties will be discussed.

Chapter 5 will show the expression, purification and crystallization conditions for two calcium sensors of GFP (7E15 and 172EF-III) designed and engineered in our laboratory.

Table 1.1: mCherry proteins (MC) and Enhanced Green Fluorescent Proteins (EGFP) worked throughout each chapter.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Purpose</th>
<th>Type</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-1</td>
<td>Express and purify</td>
<td>Mutation</td>
<td>4</td>
</tr>
<tr>
<td>MC-2</td>
<td>Express and purify</td>
<td>Mutation</td>
<td>4</td>
</tr>
<tr>
<td>MC-17</td>
<td>Express and purify</td>
<td>Grafted loop-I of CaM</td>
<td>4</td>
</tr>
<tr>
<td>MC-115</td>
<td>Express, purify, quantitative analysis</td>
<td>Grafted loop-I and EF hand motif of CaM</td>
<td>4</td>
</tr>
<tr>
<td>MC-153</td>
<td>Express, purify, quantitative analysis</td>
<td>Grafted loop-I and EF hand motif of CaM</td>
<td>4</td>
</tr>
<tr>
<td>MC-169</td>
<td>Express, purify, quantitative analysis</td>
<td>Grafted loop-I and EF hand motif of CaM</td>
<td>4</td>
</tr>
<tr>
<td>MC-187</td>
<td>Express, purify, quantitative analysis</td>
<td>Grafted loop-I and EF hand motif of CaM</td>
<td>4</td>
</tr>
<tr>
<td>EGFP 7E15 (D11)</td>
<td>Express, purify, crystallographic study</td>
<td>Mutation</td>
<td>5</td>
</tr>
<tr>
<td>EGFP172 (G1)</td>
<td>Express, purify, crystallographic study</td>
<td>Grafting EF-III of CaM</td>
<td>5</td>
</tr>
</tbody>
</table>

1.8. Significance of this thesis

The significance of this work is of great value for future researchers. The process of cell growth during reproduction is good to understand and extra cellular calcium and pH can affect this growth altering cell functions, therefore testing calcium and pH in bacterial cell *E. coli* will highlight remarkable data for the development of new cell strains commonly used for protein expression and drug manufacturing. In addition, the design of calcium binding sites in mCherry will provide further knowledge on the key factors of calcium binding and will demonstrate that our designs can be utilized in a multitude of proteins And finally, the X-ray crystallography study on calcium sensor designs will give the ability to understand the intricate properties of calcium binding and will contribute to better calcium sensor designs and knowledge of the
chromophore formation of EGFP when binding to calcium, by demonstrating the amino acids necessary for proper formation.
CHAPTER 2

2. MATERIALS AND METHODS

2.1. Competent Cell Preparation

The bacteria cell strains of *E. coli* are chemically prepared in LB medium with calcium chloride. A fresh LB no antibiotic plate is streaked with glycerol stocks of *E. coli* cells and allowed to grow overnight at 37 °C. A single colony is inoculated into 10 mL of LB (no antibiotic) and grown overnight at 37 °C. The overnight growth is transferred into fresh LB (no antibiotic) in a 100x dilution into 200 or 400 mL of media and allowed to grow to an optical density of 0.5. Samples are taken near the flame. The cells are then centrifuged at 3K for 5 min after balancing near the flame in a previously autoclaved 500 mL Oakridge centrifuge bottle. The supernatant is discarded and the pellet is resuspended in 100 mM CaCl$_2$ by gently swirling (previously autoclaved). If the cells are grown in 400 mL of media, 24 mL of CaCl$_2$ should be used. The cells are then incubated on ice for at least 30 min. The cells are centrifuged at 5.5K for 7 min and the supernatant is discarded. The pellet is resuspended in cold 100 mM CaCl$_2$ (5 mL for 400 mL of growth) and 15% glycerol (previously autoclaved) by gentle swirling. The cells are then aliquotted into autoclaved 1.7 mL eppendorf tubes and stored at -80°C for use.

2.1.1. Cloning and transformation of DNA plasmid into bacteria cells

Most of the engineered proteins studied in this thesis were engineered by Dr. Jin Zou and the graduate students Jiang Yusheng and Ada Tang. The cell strain used for transformation procedures in this study DNA was BL21(DE3). The antibiotics varied based on plasmid type based on resistance; for mCherry we used the antibiotic ampicillin (Plasmid pRSETb) and for GFP we used kanamycin (Plasmid pET28b). To begin, 50 μL of the cell strain BL21(DE3) is added into a labeled eppendorf tube, followed by the addition of 0.5 μL of DNA plasmid. The
remaining cell strain sample is labeled as a control to determine if there are other cell plasmids resistant to the antibiotic. The eppendorf tubes (protein and control) are placed in ice for 30 minutes and then in 42 °C water bath for exactly 90 seconds to allow the DNA diffuse into the bacteria cell. Following the heat shock, samples are returned to the ice bucket for 2 minutes. Next, 50 μL of Luria-Bertani (LB) media with no antibiotic is used to feed the transformed cells preventing contamination; this amount is added to the tubes and they were then placed in a shaker or incubator for 30 minutes at 37 °C. After 30 min 30 μL of the cell culture from the protein tube is transferred by pipetting to a labeled LB agar plate containing 30μg/mL ampicillin. The mixture is streaked across on the agar surface using a sterile triangle spreader.

The same procedure is followed for the control sample only containing the cell strain. Both plates are labeled and placed at 37 °C overnight gel facing up (12-15 hours). In the morning, plate with colonies of our protein is stored at 10 °C. The control plate is discarded if no colony formation, otherwise both protein and control need to be discarded. The grown protein plate can be used for 4 weeks and less than a week for protein comparison.

2.2. Protein expression media and methods for analysis

2.2.1. Luria Bertani (LB) media

Luria Bertani (LB) broth Miller medium is used for cultivation of E-coli in molecular Biology procedures. LB ingredients are:

- Peptone from casein 10g
- Yeast extract 5.0g For 1000 mL preparation
- Sodium Chloride (NaCl) 10g

To prepare LB media for inoculation and expression, we prepare 500mL and an extra 1L respectively. We wash and rinse 2 flasks (2L and 1L) with hot water brushing the flask walls really well and then rinsing with d-H₂O. Addition of 25g of LB broth with 1L of dH₂O is
followed for the larger flask and 12g with 500mL of dH\textsubscript{2}O for the small one. The flasks are autoclaved for 15 min at a temperature of 121°C.

For LB media with addition of calcium concentration, 10mM calcium chloride (CaCl\textsubscript{2}) is added up to this point. For Ca\textsuperscript{2+} ion inhibition in LB, we use 10mM of EGTA powder adjusting the pH back to pH 7.0 using sodium hydroxide (10M NaOH). The mouths of flasks are covered with aluminum foil and flasks then are autoclaved for 15 minutes at 121 °C and leave cooling for 45 more minutes (Total: 1 hour) pH 7.0+- 0.2.

### 2.2.2. Large and small scale expression of proteins

For 1L protein expression, we inoculate a single and healthy clone using a loop from the plate to a 50 mL falcon tube containing 10µL of ampicillin and 10 mL autoclaved Luria-Bertani (LB) medium. Same procedure is used if more than one mutant. The falcon tubes are then placed in the shaker to incubate overnight at 37 °C and at a speed of 220-230 RPM. Sometimes inoculations can be varied; 250 mL LB can also be used to grow one clone if more than 1L of the same protein is to be expressed. It has been noticed that due to the difference in protein-media ratio, for a lower volume of growth, the expression level came out at lower rate compared to the one with higher volume.

There are different ways to express proteins using different amounts of media; normally the ratio of inoculated media vs. media for transfer is 1:10. Different scale expressions will vary only ending protein yields but will leave the time for growth constant. The benefit for implementing small scale expressions is just for easiness and less material consumption. For large scale expression, each inoculated media is placed into the 2 L flask containing 1L LB media previously autoclaved with 100µg/mL ampicillin for expression of mCherry protein and 50µg/mL kanamycin for expression of GFP (Appendix). Followed by this, the flask is placed in
the shaker at 37 \(^\circ\)C. The optical density (OD) is checked at regular intervals to evaluate cell growth using a Shimadzu UV-1601 PharmaSpec UV-vis spectrophotometer with UV probe software (Shimadzu North America, Columbia MD). When the OD reached approximately 0.6-0.8, cells are induced with 0.2mM isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) for both fluorescent proteins (200\(\mu\)L). The structure of IPTG is shown in (Appendix). IPTG is an effective inducer in the concentration range of 100 \(\mu\)M to 1.5 mM. Shaker temperature is reduced from 37\(^\circ\)C to 25\(^\circ\)C after induction. The cells pellets are collected when the OD reaches 2.1-2.3 normally after 17-20 hours after induction.

For comparison and optimization of protein expression, we can use small scale expression. For this part, we inoculate one clone from the plate in 10 mL LB media. The inoculated media is then transferred to a 250mL LB media adding 250 \(\mu\)L ampicillin from a stock solution of 100 mg/mL AMP. The induction is performed by adding 0.2mM IPTG 50\(\mu\)L from a 1M stock solution when OD reaches 0.6-0.8. Shaker temperature is reduced from 37\(^\circ\)C to 25\(^\circ\)C after induction and cells are allow to grow for 17-20 hours as normal. The growth is spun in Oak Ridge tubes at 7 K for 20 minutes at 4 \(^\circ\)C. The supernatant is poured off and the cell pellets are scraped into a 50 mL Falcon tube. The cell pellet is stored at \(-20\) \(^\circ\)C for purification. A cell growth curve is then constructed, using the O.D(600nm) readings, and an SDS-PAGE gel is run for all aliquots taken during expression to determine if the desired protein has been expressed (Figure 2.2). In addition, fluorescence intensity emitted by chromophore is measured using the FluoStar microarray reader and a bar graph is constructed from the data collected.

2.2.3. Microplate fluorescence FluoStar reader

Samples collected during expression are taken for FluoStar measurements. The cell pellet from 1mL bacterial growth is resuspended in 200\(\mu\)L of 10 mM Tris, pH 7.4. The cells are then
added to the Corning 96 well plate with 10 mM Tris, pH 7.4 as the background. The fluorescence was then monitored by the Fluostar. To begin with the Fluostar instrument, double click the “Fluo32” shortcut on the desktop. Click Run. Go to Test Setup on the menu and click Layout. Click Create Layout. Click New, choose 96 well plate and enter a name. Select cells for samples. Choose standard, blank, and samples to be tested from the drop down menu on the left, then click in the grid on the right at the appropriate place. For example, if the standard is in the top left well, select standard from the drop down menu and then click in the top left cell (the program will highlight that cell and label it accordingly. Click OK when finished. Go to Test Setup on the menu and click Tests. Click New, well mode, select microplate brand (we use Corning, 96). Click Layout, enter the excitation and emission filters of your choice, the other factors do not need to be changed. Click OK twice. To insert the plate into the instrument, click plate out on the menu buttons at the top of the screen. The plate holder will eject, place the plate into the holder. Click plate in on the menu buttons. Click Measure on the menu buttons (looks like a stop light). Choose the profile you made from the tests list. Click Plate ID, type a filename. Click Gain. Select the entire grid or select the highest fluorescence sample, click well adjustment (the machine will measure for the gain and make the adjustments). Click OK. Click Start. When the measurement is finished, usually about 1 min, go to Results on the menu and choose Excel. The software will export your results to Excel. In Excel, double click on the filename you entered. Click Update. Choose the Evaluation spreadsheet at the bottom. The results will be displayed as raw data and as corrected with the blank, copy the spreadsheet into a new workbook to save. The spreadsheet can also be printed at this point.
2.2.4. Designed protocol to test the effect of calcium in E-coli cell strain BL21-DE3

In chapter 3, the analysis of calcium and pH on cell growth and protein expression was obtained. These mCherry and Green fluorescent protein (GFP) variants were expressed in LB media using a newer small scale expression based on methods described earlier (Figure 2.1).

Figure 2.1: mCherry WT and GFP WT protein expression using the bacteria cell strain BL21(DE3) from E. Coli. Flasks were allowed to grow for 20 hrs after induction with 0.2mM IPTG. Induction is achieved when cells reach an OD(600nm) of 0.6. Temperature is reduced from 37 °C to 25 °C after addition of IPTG.

2.3. SDS-PAGE gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS-PAGE has been used to analyze protein expression and purification based on molecular weight of protein sequence. Samples to be analyzed were mixed with SDS-PAGE sample buffer (glycerol, 1M Tris HCl buffer pH 6.8, SDS, bromophenol blue, and dH₂O) and 5% β-mercapto ethanol to avoid sulfur-
sulfur bridge formation. Mixed and denatured samples in eppendorf tubes were heated in a boiling water bath for 10 minutes. They were loaded into a SDS casting gel containing a stacking gel on top (4% polyacrylamide). A constant current of 118 V was applied and the samples were allowed to run until they migrated to the bottom of the resolving gel. The gel was stained using Coomassie brilliant blue staining buffer and 0.1% Coomassie blue R-250 methanol, acetic acid, dH2O 40:10:50 for 20 min and then distained using distaining buffer solution (25% methanol, 7% acetic acid and dH2O). mCherry containing 240 amino acids and GFP containing 238 (His-Tag included) form the molecular weight of the proteins 34KDa and 31KDa respectively.

2.4. Purification of mCherry and GFP proteins

Purification is a form to take out the protein of interest from the cytosolic site of the cells. This is performed by two methods: French Press (FP) and/or sonication.

2.4.1. Cell disruption using French Press (FP) and/or Sonication

Several methods are commonly used to physically lyse cells, including mechanical disruption, liquid homogenization, high frequency sound waves, freeze/thaw cycles and manual grinding. For E.coli cell disruption our lab uses the French Press and sonicator instruments. Cell disruption by French press (1240E CELL DIS) is used when the cell walls are too thick or simply when there is a large volume of sample. Cell pellet obtained after protein expression is dissolved with 10mL extraction buffer, which contains 20mM Tris, 100mM NaCl and 0.1% stocked titron X-100 solution and 1800 marked pressure is applied. Four repetitions are used for mCherry and GFP proteins. Sonication is the other kind of method to break cell walls, but this uses high frequency sound waves that destroy the phospholipids of the bacteria membrane and liberates the expressed protein. The solution used to dissolve the cell pellet is the same extraction buffer. The high frequency probe of the instrument is inserted in protein solution in ice and
applying the sound wave 6x for 30 seconds in intervals of 3 minutes. The protein solution is centrifuged at 17RPM for 20 minutes. The next step is to filter the protein and take it for the fast protein liquid chromatography (FPLC).

2.4.2. Methods for purification using the AKTA prime FPLC system

Purification of His-tagged EGFP is done by thawing out the cell pellet, attained from expression, and preparing the AKTA prime for Fast Protein Liquid Chromatography (FPLC) starts purification. Once the cell-protein solution has been spun, supernatant containing the protein is collected in a 50ml Falcon tube, and cell pellet is stored at -20°C. A 20μl sample of the supernatant is taken and suspended in 20μl of sample buffer with 5% β-mercapto ethanol to run a SDS-PAGE, while a small sample of the cell pellet was taken in suspended in 200μl of β-mercapto ethanol. Once the AKTA prime pumps have been cleaned with dH₂O, by running programs 8 and 9 for both pumps A and B respectively (Appendix), the column is then positioned and loaded with 0.1M nickel sulfate (NiSO₄), using program 10 (Appendix) use for binding. Once loading is complete, the free nickel is washed from the column, using the same program 10 that goes to the column with de-ionized water twice (5ml/min for 10 min). The protein sample previously been filtered is then injected into the system for binding to nickel in column, accomplished by running program 6, used only for sample injection. Pump A is dip into a filtered buffer A (50mM PO₄ pH7.4 and 250mM NaCl) and program is ran for 1 hour. Once all the protein has been bound to the column the sample is eluted, placing pump B into Buffer B (same buffer A plus 0.5M imidazole), by running program 7, which was set for elution of the sample. Once eluted, samples are taken from selected fractions based on fluorescent color. The best three fractions are tested by SDS-PAGE to determine protein purity (20μL protein + 20μL sample buffer). After protein elution, the column is cleaned, using a 100mM EDTA and 1M
NaCl solution, by running program 10. The EDTA solution is removed from the column by running program 10 again, but placing pump A into de-ionized water (dH2O). The pumps are finally cleaned using de-ionized water. The purified protein sample is dialyzed over a 3-day period to using 2L of 10mM Tris and 1mM DTT buffer pH 7.4 exchanging it 3x to remove the imidazole. The tubes with smaller amounts of protein, indicated in the SDS-PAGE gel, are then combined into centrifuge tubes and concentrated down. The protein samples, once concentrated, are then dialyzed in the same manner. Once dialysis is completed UV-Vis at 350nm is run for the dialysis solutions, taken after each day, and the protein sample.

![Image of protein purification method](image)

**Combining 3 Fractions ~ 40uM (18mL)**

Figure 2.2: Sketch illustrating protein purification method by 6x-His-Tag affinity column and gel filtration column.

Upon completion of purification followed by dialysis, UV-Vis is run, between the wavelengths 600 nm and 200 nm. The UV-Vis spectrum indicates that there is protein present in the elution. The concentration of protein recovered from purification is calculated approximately 90 µM in 8 ml of solution. Also, UV-Vis was run for the dialysis solutions after days one, two,
and three. The absorbance at 280 nm, indicating the presence of imidazole, decreased after each day of dialysis. In addition, UV-Vis is also run for varying imidazole concentrations, which is used to construct a standard curve, to quantify the amounts of imidazole dialyzed from the protein samples. After day one of dialysis the calculated imidazole concentration in solution is about 0.012M. After the second and third days of dialysis the imidazole concentration will be undetectable.

2.5. pKa Measurements of EGFP and EGFP Variants

To measure the chromophore pKa of mCherry variants, separate samples were made at each pH to be measured and the absorbance and fluorescence of the samples were examined. Various buffers were utilized to maintain the pH at each desired value, which are listed in table 2.1. The stock buffers were made by Dr. Jin Zou in this laboratory for everyone’s use with aliquots taken by each lab member. The protein concentration was ~10 µM for each mutant. A baseline was run for each pH with the appropriate buffer before the sample at that pH was measured. The absorbance was measured from 600 nm to 200 nm so all protein peaks would be included. The cuvettes were cleaned between pH samples with 1% Liqui-nox to remove any protein. The pH was measured for each sample after the spectra were acquired. For fluorescence, the samples for absorbance were diluted 10x (in duplicate) with the appropriate buffer for a final concentration of ~1 µM. The samples were allowed to equilibrate overnight at 4 °C. The fluorescence was measured with the excitation at 586 nm, the excitation slit width at 1.5 nm, and the emission slit width at 2 nm. The emission was scanned from 350 to 650 nm. The sample at pH 9 was measured first so the slit widths could be set for optimal intensity, and the samples were measured with decreasing pH. The cuvettes were cleaned with 1% Liqui-nox.

Table 2.1 A list of the buffers utilized for the pH dependence measurements.
<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Acetate (CH3COOH)</td>
<td>2.09</td>
</tr>
<tr>
<td>0.5 M Acetate (CH3COOH)</td>
<td>4.0</td>
</tr>
<tr>
<td>10 mM Acetate</td>
<td>4.5</td>
</tr>
<tr>
<td>10 mM MES (2-Morpholinoethanesulfonic acid)</td>
<td>5.0, 5.2, 5.7, 6.0</td>
</tr>
<tr>
<td>10 mM PIPES (1,4-Piperaezinediethanesulfonic acid)</td>
<td>6.5, 7.0</td>
</tr>
<tr>
<td>10 mM Tris [Tris (hydroxymethyl) aminomethane]</td>
<td>7.4, 8.0, 8.5, 9.0</td>
</tr>
</tbody>
</table>

Figure 2.3: Curve fitting for titration points during analysis. B initial point, A final point. pKa=m3.

The pH value where the titratable group is half-protonated is equal to the pKa if the titration curve follows the Henderson-Hasselbalch equation. Most pKa calculation methods silently assume that all titration curves are Henderson-Hasselbalch shaped, and pKa values in pKa calculation programs are often determined in this way [33].
2.6. Protein crystallography screening for GFP calcium sensors

The Vapor Diffusion (Hanging Drop Method) was used for the analysis of both sensors of GFP: D11 and G1. The buffer preparation and diffusion principle is shown (Figure 2.4).

1. Pure protein: > or = 10 mg/mL
   - Binding Affinity (His-Tag)
   - Gel Filtration column

2. Well solution:
   - Precipitant: PEG (3350) or 4000
   - Buffer: HEPES
   - Additive: MgCl₂, β-mercaptoethanol

3. Suspended drop:
   - 2µL Protein sample
   - 2µL of well solution.

4. Sealed glass cover slip

*** Everything Must be Filtered

Figure 2.4: Hanging drop method for protein crystallization

Pure protein after His-Tag and gel filtration columns needs to be obtained in concentrations higher or equal to 10mg/mL. Stock solution of polyethylene glycol (PEG 3350 or 4000) is prepared in a 50mL falcon tube 50% PEG concentration, 25g PEG3350 with dH₂O to the 50mL mark. In one clean bottle, 1L of HEPES buffer is prepared at 500 mM pH 7.0. Five falcon tubes with 50mL of HEPES are filled changing pH from pH’s 6-8. In addition, 500mM MgCl₂ and 10mM β-mercaptoethanol are prepared in 50mL volumes. All buffers and protein need to be filtered using 0.45µM filters. Next, a microplate reader is used for growing the protein crystals. Using a syringe filled with grease, we surround the mouth of each well with a delicate circle of grease to enclose the protein hanging-drop after placing the glass cover slip. A crystal screening
is developed first by changing PEG concentration and pH’s (Figure 5.9). The initial crystal screening is obtained placing a total 1mL volume combining 50mM HEPES buffer pH 6.5-8.0 in rows 1 to 4, PEG 3350 (12-17%) in increasing order by column, 50 mM MgCl₂ and 1mM β-mercaptoethanol. For example, in well screened A1 12% PEG 50mM HEPES pH 6.5, 50 mM MgCl₂ and 1mM β-mercaptoethanol, we add 240µL of 50% PEG, 100µL of 500mM HEPES pH 6.0, 100µL of 500 mM MgCl₂, 100µL of 10mM β-mercaptoethanol, and 460µL of dH₂O. Followed by this, on a cover slip we mix 2µL of the protein with 2µL of buffer from well. The cover slip is placed in the well hanging the protein-buffer drop inside the microplate well. Microplate is stored at 25°C until crystal growth is observed.
CHAPTER 3

3. THE EFFECT OF CALCIUM AND PH IN BACTERIAL CELL GROWTH AND EXPRESSION OF FLUORESCENT PROTEINS AND OTHER VARIANTS

3.1. Introduction

3.1.1. Role of calcium and pH in eukaryotic and bacteria cell reproduction

Escherichia-Coli, a type of organism in nature that can be cultured easily under different conditions has the ability to grow fast under normal conditions. It is used in different laboratories by researchers for a large number of genetic studies. This microorganism has been one of the widely applied for the production of many chemicals, drugs, and proteins in techniques in the area of research. E. coli bacteria as mentioned before can be easily cultured in either as colonies on agar plates containing nutrient medium or in suspension liquid media LB (Luria Bertani Broth media) [34] [35]. In this laboratory, both techniques for growth (plate and LB) of E- coli were commonly used to grasp the basis of common growth performed in the lab.

Bacteria reproduce by binary fission and their growth increases at a rate of 1000 cells per minute at a temperature of 37°C. In liquid rich medium, bacteria E-coli can increase in number until they reach a peak where either the media is insufficient for development or an accumulation of waste products is produced when time passes. The specialized organisms prevent the continual replication of cells when they feel the necessity of more food. This peak point is called the “stationary phase,” or the point when saturation or high concentration of cells is produced with time [36] [37] [38]. The cells undergo a physiological change that allows survival instead of growth. The “exponential” or logarithmic phase is the time when bacteria grow exponentially reaching a peak where they can be transcribed taking the lac-repressor by induction using IPTG (isopropyl β-D-1-thiogalactopyranoside). The step is promoted by previously cultured cells from the “stationary phase” diluted to new media LB. Most physiological experiments are conducted
using rapidly dividing log phase cultures. Cell growth can be measured by following the increase in absorption of a culture at 600 nm using a UV-vis absorbance spectrophotometer. A culture with an absorbance of 1.0 at 600 nm contains approximately $2 \times 10^8$ cells/mL.

3.1.2. The effects of calcium and pH in bacteria cell reproduction

Among several factors that are important to bacteria cell growth and their capability in expression of proteins, calcium and pH were the most important ones. Although bacterial cells do not have the ER and nucleus, calcium ions were observed to participate in the regulation of several aspects of cell division and cell growth. Few systematic studies have been devoted to investigating the role of calcium as an intracellular messenger in prokaryotes. From a report in the investigation on the potential involvement of calcium in signaling in Bacillus subtilis, a Gram-positive bacterium, it is shown that B. subtilis cell tightly regulate intracellular calcium levels[39]. This homeostasis can be triggered by an external stimulus such as hydrogen peroxide, pointing to a relationship between oxidative stress and calcium signaling. In addition, B. subtilis growth appears to be intimately linked to the presence of calcium, as normal growth can be immediately restored by adding calcium to an almost non-growing culture in EGTA containing Luria broth medium (Figure 3.2). Addition of iron or manganese also restores growth, but with 5-6 h delay, whereas magnesium did not have any effect. In this reported analysis, the experiment on cell growth using EGTA as calcium and other metal chelating agent reveals that the increasing addition of EGTA to a 10mM level significantly reduced cell growth. In this study, the addition of different concentrations of EGTA to LB media was performed when the cells grew for 1.5 hours. There is no evidence pH in media was controlled during EGTA addition.

On the other side, pH was reported to be a major factor during cell growth. From a study developed by Presser et al, the growth rate responses of E.coli M23 (a nonpathogenic cell strain)
to pH and lactic acid concentrations were developed (Figure 3.1). The results using a range of pH 2.71 to 8.45 and fixing specific values for lactic acid showed that *E. coli* grew at pH 4 but not pH 3.7 and was unable to grow in the presence of 8.32mM and higher[40]. Decreases in pH cause only relatively small decreases in the growth rate, except when the limiting pH is approached, at which a more significant decrease in the growth rate occurs. The pH response has been described by other authors as a symmetrical parabolic curve [41]. It has been reported that *E.coli* has great capacity to evolve to be more resistant to pH changes. For example, *E. coli* O157:H7 has been found to grow on foods at pHs greater than 4 (fruit, vegetables, and beef) while it is not able to proliferate in foods at pHs less than 4[40]. To date when pH was reported to be factor on microbial growth it is not clear how pH will effect on protein expression.

Figure 3.1: A. Growth rate (1/generation time [in minutes]) of *E. coli* M23 in the absence of lactic acid versus hydrogen ion concentrations (micromolar). The line was obtained by linear regression. B. Growth rate (1/generation time [in minutes]) of *E. coli* M23 versus un-dissociated lactic acid concentrations (mM) for experimental data for 25 (squares), 50 (circles), and 100 (triangles) mM total lactic acid concentrations. The line was obtained by linear regression.
Figure 3.2: A. LB medium containing 0 mM (♦), 1 mM (▪), 5 mM (Δ) or 10 mM (•) EGTA was inoculated (at a 1/100 dilution) with BS168 grown overnight in LB medium. Bacterial growth at 37°C was followed by measuring absorbance at 600 nm (OD600) as a function of time. Similar curves were obtained when the experiment was repeated. B. LB medium containing 0 mM, 1 mM, 5 mM or 10 mM EGTA was inoculated (at a 1/100 dilution) with mCherry WT grown overnight in LB medium. Bacterial growth at 37°C was followed by measuring absorbance at 600 nm (OD600) as a function of time.

3.1.3. Hypothesis and objectives of this study

Based on previous studies on factors contributing cell growth and protein expression, we have developed a study to test calcium effects and pH on the cell strain BL21(DE3). In this chapter we want to address the question: what are the effects of calcium and pH in bacterial cell growth and protein expression. In order to answer this question two main hypotheses have been thought using our general lab applications. The first hypothesis is that E. coli cell growth may be altered by changing pH and calcium in the medium. The second hypothesis determines that calcium and pH change may alter protein expression. E. coli cell growth and its capabilities in expression of the red fluorescent protein: mCherry were tested in 10mM extra calcium addition in LB medium and also in 10mM EGTA (calcium chelating agent) with and without controlling pH to test the different changes.
3.2. Cell growth analysis testing the effect of calcium and pH in bacteria cells

To test calcium effect and pH change, the cell strain BL21-DE3 of E-coli will be used. In the first hypothesis we think that by enhancing and inhibiting cell growth with 10mM CaCl$_2$ and 10mM EGTA respectively controlling and not controlling pH as a cofactor, bacteria cells would uptake calcium from media for their growth and rapid development. In the other hand calcium inhibition by EGTA will slow down the rate at which these normally grow. The second hypothesis is that protein expression level would increase in cells present in calcium containing media, while no high expression would occur in calcium cell inhibition with EGTA.

3.2.1. Choosing the conditions and cell strains

There is great number of cell strains in the market for protein expression. The selection of a particular cell strain that will result in optimal cell growth and expression of desired proteins needs to be tested. The most widely cell strain used for protein expression is BL-21. This cell strain minimizes protein degradation by minimizing the expression of protease enzymes. Red and green fluorescent proteins such as mCherry and GFP are best expressed using the cell strain BL-21(DE3) and BL-21(pLysS). Both cell strains have the lactose analog promoter system and protein expression can be induced by IPTG. Furthermore, bacterial cells can be easily lysed using sonication or French press (FP). Normally, different cells are grown using two types of media (LB and SV media) at different temperatures and IPTG concentrations for induction. Different cell strains under the same conditions for a period of ~20 hours to compare best protein expression yield results by running the samples after induction overnight in sulfur dodecyl sulfate polyacrylamide (SDS) gel electrophoresis. Thus, the cell strain BL21-DE3 was used for the expression of both variants of designed calcium binding fluorescent proteins for this study.
3.2.2. Inhibiting calcium with EGTA in bacteria cell growth without controlling pH

Similar to the reported study on B. subtilis growth[39], we first designed an analysis in bacteria cells E-coli in the presence of 10mM EGTA LB and enhancing the calcium level in media by adding 10mM calcium (see methods). Normal cell growth in LB media without the addition of calcium and EGTA was used as a control. Since in the previous reported study, 10mM EGTA was enough to inhibit cell growth in bacteria cells B. subtilis, the same concentration was in our study for inhibition of E-coli. The OD at 600nm was taken at different times for cell growth (Figure 3.2). The three media expressing the protein mCherry wild type (WT) and GFP wild type (WT) and then with other protein variants also were tested (Figures 3.3 and 3.4). The yield of cell pellet was examined after cell expression (Figure 3.5).

To remove Ca$^{2+}$ in the medium, the LB media for culturing cells was pretreated with EGTA, a commonly used Ca$^{2+}$ or La$^{3+}$ chelating agent an ion which competitively inhibits Ca$^{2+}$ movement through plasma membrane channels (Figure 3.2). The induction of mCherry and GFP protein expression in the presence of EGTA in LB was at the time the control (LB only) reached the OD(600nm) of 0.6. pH drops upon the addition of 10mM EGTA to LB media from pH 7 to pH 3.9. It is of great importance to highlight that the pH was not controlled in the first analysis following the same procedure used in the growth analysis of B. subtilis.

In (Figure 3.3), pH was not controlled; the OD (600nm) represents the cell growth for both fluorescent proteins mCherry and GFP wild type (WT). Bacteria cell growth in normal LB media and with addition of 10mM CaCl$_2$ in LB resulted in very similar growth. After inducing the cells with 0.2mM IPTG, there was no big effect in cell growth when adding extra 10mM calcium concentration in LB for both fluorescent protein expressions. On the other hand, cell growth in the presence of 10mM EGTA in mCherry decreased significantly after 4 hours and collected.
after overnight. This effect seemed to be larger for mCherry (54%) than for GFP (13%). Figure 3.3b shows the results for a similar experiment using other variants of the proteins mCherry and GFP.

As observed from the graphs from figure 3.3b, we obtained non-significant changes for cell growth in media containing extra addition of calcium for mCherry variants, and the same happens in cell growth for LB media containing extra calcium and the control for GFP variants. Evidently, the presence of EGTA affects cell growth in all variants with a decrease in cell growth of 20-25% compared with the control media.

The percent yield for the cell growth is standardized as a mean value from repetitive trials expressing different variants of mCherry and GFP. Results show that cells grown in media containing 10mM CaCl$_2$ were not significant based on statistical analysis. In the cells grown in EGTA media, our reported results suggested that EGTA inhibits cell growth and protein expression at the end of the expression; the cell pellet was reduced by approximately 34% when comparing it to the control. Since pH decreased from & to 3.9, this could be a possible reason for change.
Figure 3.3a: OD (600nm) at different times for the protein expression of mCherry WT and GFP WT. Two flasks (400mL) of LB media were pretreated with 10mM EGTA, Only LB, and 10mM Ca\(^{2+}\) addition respectively. 10mL of culture media (BL21DE3) with the mCherry gene was transferred to each flask and allowed to grow overnight after induction with 0.2mM IPTG. Temperature was reduced to 25\(^{\circ}\)C after induction.

Figure 3.3b: OD (600nm) at different times for the protein expression (5x dilution OD >1) of two of mCherry and two GFP variants. Three flasks (400mL) of LB media were pretreated with 10mM EGTA, Only LB, and 10mM Ca\(^{2+}\) addition respectively. 10mL of culture media (BL21DE3) with the mCherry gene was transferred to each flask and allowed to grow overnight after induction with 0.2mM IPTG. Temperature was reduced to 25\(^{\circ}\)C after induction.
Figure 3.4: A: % yield of cells (BL21 DE3) E-Coli obtained after the expression of 3 flasks containing 400 mL of LB media using the protein gene mCherry (~25hrs). Higher yield occurs in the flask containing LB + 10mM Ca\(^{2+}\) follow by LB only and LB + 10mM EGTA respectively. B: Same as left except that for this the GFP protein gene was expressed in the three different media. Higher yield occurs in the flask containing LB + 10mM Ca\(^{2+}\) follow by LB only and LB + 10mM EGTA respectively.

3.2.3. Inhibiting calcium with EGTA in bacteria cell growth controlling pH

From an understanding using EGTA as a calcium chelating agent, we saw that the reduction in pH could play an important effect for cell growth. For the next analysis, we designed a similar study as the one described in section 3.1 but this time stabilizing pH values after autoclaving the LB media containing 10mM CaCl\(_2\) and 10mM EGTA. One sample containing 10mM EGTA was left unchanged and pH showed a value of 3.93. In (Figure 3.5) the growth of the E-colı cell strain BL21-DE3 can be observed. In the bacteria cell B-subtilis168, the inhibition is greater reaching to an almost no level of growth after 8 hours. To test the hypothesis in which pH is a key factor controlling cell growth, the expression of mCherry protein was repeated.
Figure 3.5: Expression of 4 L of mCherry WT (250mL inoculation with one clone of the gene). Each individual flask for inoculated transferred contained LB + 10mM Ca\(^{2+}\), LB only, LB+ 10mM EGTA (pH no changed), and LB+10mM EGTA (pH 7.0) respectively. 1mL cell growth aliquot was taken before induction (BI) with 0.2mM IPTG, 2hr, 4hr, and Overnight after induction (AI) for OD (600nm) measurements.

It is seen by OD (600nm) measurements that BL21-DE3 cells were not completely inhibited by the addition of 10mM EGTA to LB media (pH 7) comparing it with the sample with pH unchanged (pH 3.9).

From figure 3.5, we can see that the inhibition of metals on cell growth with EGTA maintaining pH stable (pH 7) was less than without controlling pH after expressing overnight under the similar conditions. LB without any addition of calcium or EGTA produced the best cell growth; this is followed by the media containing extra addition of 10 mM CaCl\(_2\).

Inhibition of cell growth occurred in presence of 10 mM EGTA; 25% decrease (pH 7) and 35% decrease (pH ~4). Although cells did not reach the maximum of the cell decay during expression, pH effect change was detected in cell growth by having a decrease of 10% after expression 8 hrs
when this is not controlled. Extra calcium levels in media also reduced the cell growth by almost 7% in this experiment.

3.3. The effect of calcium and pH on the expression of fluorescent proteins

Protein expression level was also analyzed in this experiment. In order to monitor the chromophore formation of the fluorescent protein in the bacteria cell BL21-DE3, fluorescence emission of samples at 590nm and 420nm for mCherry and GFP respectively were obtained during expression using fluorescence microplate readers and a FluoStar instrument (Figure 3.6A). In addition, SDS-PAGE was applied to monitor protein expression at different times before and after induction with 0.2mM IPTG (Figure 3.6B).

Figure 3.6: A. Fluorescence intensity from 1mL aliquot obtained during expression. To the cell pellet, 200mL of 10mM Tris buffer pH 7.4 was added for analysis in micro-plates. Reference samples contained only 200 mL Tris buffer. Similar graphs were obtained when analysis was repeated several times. pH in media was not controlled. B. SDS gel showing the bands for the expression of the protein mCherry WT (MW: 34KDa). Cell pellet from 1mL aliquot sample of cell culture was mixed with 250µL of sample buffer (5% 2-mercapto Ethanol) and boiled 5 min. 4 µL of protein marker and 10 µL of sample was loaded.

During protein expression in different media containing addition of calcium and EGTA without controlling pH factors, we were able to obtain the following results: The extra addition
of calcium in LB media showed higher fluorescence in mCherry wild type than the control (LB only) by almost 40%. Chromophore formation during the protein folding in expression achieved a successful increase that when inhibiting cells with EGTA. Indeed, 10mM EGTA addition to the LB media for protein expression reduced the fluorescence by almost 20% when these samples were analyzed.

We were able to detect cell expression by measuring fluorescence intensity under the same methods used in previous figure (Figure 3.3). Enhancing LB media with 10mM calcium increased fluorescence intensity after expression overnight by almost 11%, while the protein expression with the metal inhibition of EGTA (pH4) decreased by 82%. No such decrease occurred when controlling pH after addition of EGTA (pH7) during cell growth (5% decreased).

Figure 3.7: Fluorescence intensity from 1mL aliquot obtained during expression. To the cell pellet, 200mL of 10mM Tris buffer pH 7.4 was added for analysis in micro-plates. Reference values only 200 mLTris buffer. pH controlled (pH 7) and not controlled (pH 4).
3.4. Conclusions and significance

We have studied the effect of the ethylene glycol-bis (beta-amino ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) as well as extracellular calcium concentration in LB media on the growing cell line of E-coli (BL-21DE3). This work has been compared with a previous report by from the journal by Marie-Laure Herbaud studying B. subtilis growth with EGTA. When increasing the concentration of EGTA to a 10mM level significantly reduces cell growth. Unfortunately, there is not such evidence that pH conditions in media were maintained upon addition of EGTA. It is important to recognize that the addition of EGTA results in the lowering in pH up to 3 scale units. For example, 10mM EGTA in 1L LB medium (pH 7.0) lowers the pH to 4. The addition of this EGTA concentration to the media without controlling pH decreased bacteria cell (E. coli) growth by 37%. The chromophore fluorescence of mCherry decreases 97% after overnight expression.

Stabilizing the pH 7.0 from normal LB with sodium hydroxide (10 M NaOH) after the addition of 10mM EGTA decreased cell growth by 25%, a lower decrease comparing it with not controlling pH. However the chromophore fluorescence of expressing mCherry reached to wild type-like with a slow maturation rate. The addition of 10mM calcium addition had no significant effect on cell growth but it produces 15% increase in protein expression (chromophore formation). Overall pH plays an important role for E.coli cell growth and protein expression (mCherry), while extra calcium increases the fluorescent intensity at the end of the expression.

In the analysis of E-Coli cell growth we were able to obtain data with and without controlling pH. The cell strain BL-21(DE3) was selected due to the fact that it tightly regulates protein expression using their lactose analog promoter system, which can be induced by IPTG. In addition, both red and green fluorescent proteins such as mCherry and GFP are well expressed.
using and BL-21DE3. The bacteria cell walls can be easily broken to purify the proteins using sonication and/or French press (FP).

We have shown that addition of calcium does not result in a significant increase of *E. coli* cell growth. On the other hand EGTA significantly decreases cell growth. It is known that EGTA can inhibit many other metals other than calcium ions such as Fe$^{3+}$, Ln$^{2+}$ Mg$^{2+}$, Mn$^{2+}$ that are also important for cell reproduction and growth. Decrease in cell growth was observed with and without control of pH in the media. When pH of the media was not controlled, the decrease rate in cell growth after expression overnight is greater than the one maintaining pH stable.

In the second study examining the effect of calcium and pH on protein expression level, we have observed that low protein expression in the presence of EGTA, more fluorescence signal suggests that more properly formed protein and high level of expression occurred when expressing the protein in LB media containing higher levels of calcium. The EGTA significantly decreased chromophore formation of mCherry without control of pH (pH~4), while maintaining the pH at 7.0 resulted in some improve of formation of chromophore. However, that is still lower than the control media without addition of EGTA.

In summary, pH is an important factor contributing to protein expression and cell growth. The addition of calcium in cell medium does not result in significance effect on cell growth possibly due to residue calcium in cell medium is enough for cell growth. Further study is needed in this analysis.
CHAPTER 4

4. MCHERRY FLUORESCENT PROTEIN AS CALCIUM BASED SENSORS

4.1. Introduction

4.1.1. mCherry protein and its applications

mCherry next to other mfruit fluorescent proteins come from different mutations derived from the original tetramer dsRed or Red Fluorescent Protein found in the coral Discosoma sp.[42] (Figure 4.1). The monomer mRFP1 is the antecessor of mCherry and it has been proved widely it is possible to further improve its optical properties. As mentioned in chapter 3, mCherry has high qualities for this improvement. One research goal in Dr. Jenny Yang’s lab is to develop calcium sensors using mCherry as host novel protein. From a design approach followed by either mutagenesis or grafting, several mCherry have been modified in different variants. Class one is by insertion of the loop-I and/or the EF hand motif I of calmodulin (CaM-I) into different locations in the protein sequence and class two is by the addition of calcium binding sites into the protein by direct mutagenesis (Figure 4.2). In tables 4.1 and 4.2, there is a list of variants that have been mutated or grafted with calcium binding sites in our laboratory.

In order to understand the calcium binding mechanisms of mChery variants, it is important to analyze and compare different aspects of protein mechanisms. These mechanisms can be determined by protein expression: comparing chromophore formation and SDS-PAGE, purification: measuring purity and percent yield, and fluorescent key determinants such as molar extinction coefficient, quantum yield, brightness and pKa.
Figure 4.1: Structure, properties and emission and excitation peaks of mCherry fluorescent protein.

<table>
<thead>
<tr>
<th>Class</th>
<th>Protein</th>
<th>Source laboratory (references)</th>
<th>Excitation(^\text{a}) (nm)</th>
<th>Emission(^\text{a}) (nm)</th>
<th>Brightness(^\text{a})</th>
<th>Photostability(^\text{a})</th>
<th>pKa</th>
<th>Oligomerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat-red</td>
<td>mPlum(^b)</td>
<td>Tsien (5)</td>
<td>590</td>
<td>649</td>
<td>4.1</td>
<td>53</td>
<td>&lt;4.5</td>
<td>Monomer</td>
</tr>
<tr>
<td>Red</td>
<td>mCherry(^c)</td>
<td>Tsien (4)</td>
<td>587</td>
<td>610</td>
<td>16</td>
<td>96</td>
<td>&lt;4.5</td>
<td>Monomer</td>
</tr>
</tbody>
</table>

Figure 4.2: Two classes of engineered fluorescent proteins designed to bind calcium ions. 1. Grafting the calcium binding sequence of CaM (Loop-I and EF motif) into mCherry. 2. Mutations to develop calcium binding pockets in mCherry.
mCherry has been used for some applications in research; one is that it has been applied as a fluorescent fusion protein that can bind to different proteins. Scientists have been able to fuse mCherry with proteins like actin and tubulin [43]. Proteins, that once were invisible, are now viewed and quantitatively analyzed fusing them with mCherry and using Biological quantitative techniques. Due to the fact the protein acts as an efficient fluorescent energy acceptor for commercially available quantum dots, it can be detected using fluorescence resonance energy transfer (FRET) techniques [44]. This fluorescent protein is recognized to be useful in many other applications such in fluorescence recovery after photo bleaching (FRAP) and fluorescence lifetime imaging microscopy (FLIM) [45]. Two classes of engineered mcherry based calcium sensors have been introduced in our laboratory: class 1 and class 2 (Figure 4.2) as explained before. Class 1 design has been used to test the ability of the calcium binding site of CaM into mCherry. As observed in (Figure 4.3C), the calcium binding loop of CaM and the complete E-I-F hand of CaM have been inserted into residues H17-D18, D115-G116, E153-G154, D169-G170, and V187-Q188 as pointed in by the blue dots in the protein sequence.

Figure 4.3: A. 3D structure EF hand of CaM: Two α helices that are connected by a short loop region in a specific geometric arrangement constitute a helix-turn-helix motif of CaM. B. Grafted mCherry with EF
hand of CaM.C. The calcium-binding residues are numbered and underlined in the loop; these are present in many proteins whose functions are regulated by calcium.

### 4.1.2. Purification techniques designed for red fluorescent proteins

Red fluorescent proteins like mCherry which emit fluorescence in the 610nm wavelength region in the visible spectrum have been purified using different methods. One common technique is by utilizing their inherent copper binding property. Immobilized copper ions yield a single strong band corresponding to purified red fluorescent proteins on the SDS-PAGE gel [46]. This purification achieves efficiency higher than 95%. Others perform purification by using Talon metal affinity resin. Elute taken from this is further purified by gel filtration. Even other common procedures for larger amounts of proteins have been developed. The use of open columns or FPLC equipment with resins that can be used at high pressure: like Ni-NTA superflow resin, metal affinity or chelating sepharose fast flow or Ni Sepharose High Performance or Ni-NTA His-Bind or metal chelate resins are new techniques offered in the market.

In general, there are many tags and systems for purification for red fluorescent proteins and the implementations of these will vary upon the time, efficiency of system and other specificities of the protein. For our designs, we use Ni$^{2+}$ sepharose that will bind to the His-Tag on the N-terminus of the sequence. The use of Fast Protein Liquid Chromatography (FPLC) equipment allows greater operational flexibility and simple optimization.

### 4.1.3. Quantitative analysis of engineered proteins

For quantitative analysis of engineered fluorescent proteins, it is important to determine the molar extinction coefficient ($\varepsilon$), quantum yield ($\Phi$), brightness and pKa. These parameters provide information about chromophore formation, the attribution of protein environment and further development of calcium sensors and other Biosensors.
4.1.4. Molar Extinction Coefficient of chromophore in proteins

The molar extinction coefficient ($\epsilon$) of a protein at 280 nm depends almost exclusively on the number of aromatic residues, particularly tryptophan, and can be estimated from the sequence of amino acids [47]. If the extinction coefficient is known, it can be used to determine the concentration of a protein in solution based on the Beer-Lambert law:

$$A = \epsilon IC$$

Equation 4.1

- Where $A$ is absorbance,
- $\epsilon$ is the molar absorption coefficient ($M^{-1} \text{cm}^{-1}$),
- $I$ is the path length (cm), and $C$ is the protein concentration (M).

The extinction coefficient of our protein variants can be determined by comparing the excitation absorbance peak of the chromophore at wavelength 586nm with absorbance of the protein at wavelength 280nm. A high $A(586)/A(280)$ ratio represents better excitation of the protein chromophore than a higher extinction coefficient. Measuring the protein absorbance at these two peaks as a function of protein concentration, we can predict the value of our mCherry variants. To synchronize reported extinction coefficient values, the experimental extinction coefficient obtained from the protein variant is compared with the one for the protein wild type as a control. Using the equation 4.2, we can determine extinction coefficients of our mCherry protein variants.

$$
\epsilon_{p,586nm} = \epsilon_{p,280nm} \left( \frac{A_{p,586nm}}{A_{p,280nm}} \right)
$$

Equation 4.2
In which $\varepsilon_{p, 586\,\text{nm}}$ is the extinction coefficient of the protein variant to be found, $\varepsilon_{p, 280\,\text{nm}}$ is the extinction coefficient of mCherry protein counting aromatic residues at 280 nm and $A_{p, 280\,\text{nm}}$ and $A_{p, 586\,\text{nm}}$ represent the absorption intensities at 280 nm and 586 nm that can be found experimentally. In this chapter, a report of $\varepsilon$ values is determined for some designed mCherry variants that keep the red fluorescence after expression.

4.1.5. Quantum Yield and Brightness

The quantum yield ($\Phi$) of a protein is defined as the number of photons emitted as fluorescence, divided by the number of excited states that were produced in the excitation [48][49]. In other words, it is the fluorescent light emitted by the protein divided by the absorbance of light of the protein. A quantum yield ratio of zero value means no fluorescence, while a quantum yield ratio of 1 means 100% fluorescence. As described by equation 4.3, we can determine the quantum yield for mCherry proteins by measuring the emitted fluorescent intensities at 610nm and the absorbance of the chromophore at 586nm at different protein concentrations. In this work, we will used mCherry WT as a control to calculate quantum yield of other variants. By calculating both the molar extinction coefficient and quantum yield of a specific variant, we can obtain the brightness of that protein by using equation 4.4, which is the product of the two values. Brightness is defined as a visual perception in which a source appears to emit or reflect a given amount of light. In other words, brightness is the perception elicited by the luminance of a visual target [48][49]

$$\varphi_p = \varphi_r \left( \frac{F_p}{A_p} \right) \left( \frac{F_r}{A_r} \right)$$

Equation 4.3
\[ B_{p(586nm)} = \varepsilon_{p(586nm)} \cdot \varphi_{p(586nm)} \]  

Equation 4.4

4.1.6. pKa of chromophore

pKa is another factor to consider when working with an enzyme or protein. Buffers used for analysis of fluorescent proteins need to be prepared based on specific values of pKa of chromophore. Protein pKa calculations are used to estimate the pKa values of amino acids as they exist within the protein. These calculations complement the pKa values reported for amino acids in their free state, and are used frequently within the fields of molecular modeling, structural bioinformatics, and computational biology [33]. pKa values of amino acid side chains are of great importance in defining the pH dependent characteristics of a protein such as activity and stability. We can calculate the pKa of a protein by using the fitting plot equation 4.5 after a protein titration.

\[ Y = \frac{A \cdot 10^{-pH} + B \cdot 10^{-pKa}}{10^{-pH} + 10^{-pKa}} \]  

Equation 4.5

In which, Y is the fitting curve equation for the protein titration plot

A is the initial pH value in the titration

B is the final pH value in the titration.

4.1.7. Objectives of this study

The major goal in this chapter is to analyze different variants of the protein mCherry that can function as metal sensors for the study of calcium. The first objective of this study is to identify optimal conditions for expression and purification of engineered proteins and to avoid proteolytic cleavage. The second objective of this chapter is to perform quantitative analysis of protein
variants with engineered metal binding sites. We will determine the extinction coefficient, quantum yield, brightness, and pH of the engineered fluorescent probes.

4.1.8. Questions to be addressed

- Is the grafted Ca\textsuperscript{2+}-binding sequence of CaM affecting expression and optical properties of the engineered fluorescent proteins?
- Can protein variants of mCherry be diligently purified?
- Do fluorescent mCherry variants develop higher or lower extinction coefficient, quantum yield, brightness, and pH values when grafting and/or mutating the protein sequence?
- Is calcium inducing the red fluorescence in mCherry protein variants?

4.1.9. Engineered fluorescent proteins

In the past, Dr. Jin Zou started analyzing mCherry as an optimal fluorescent protein to develop calcium sensors. He started designing new calcium binding probes grafting calcium binding sites that would fuse to the fluorescent protein and would induce red fluorescence as calcium was bounded. He proposed to engineer mCherry variants grafting the calcium binding motif of CaM into the sequence at different locations. At first, he was successful in grafting the CaM motif in location K115, E153, D169, and V187. From then on, he designed new locations to insert the CaM sequence motif and experiment with the ones that obtained fluorescence after expression. He group these set of variants: class 1. Right after, the graduate student Jiang Yusheng started creating calcium binding pockets within the sequence of mCherry, he designed two calcium binding pockets which were grouped in class 2 next to mutations to prevent cleavage in mCherry (direct mutagenesis). A summary of mCherry variants from class 1 and 2 designed is represented in (Figure 4.4).
Figure 4.4: Grafted and mutated locations of mCherry for the designing of new calcium sensors. (Dr. Jin Zou, Jiang Yusheng, and Malcom Delgado).

4.2. Expression of mCherry variants: Class 1 and class 2

For expression of mCherry variants, the plasmid pRSET-B is used for cloning of the protein. The restriction sites of the plasmid are represented in (Figure 4.5) to indicate the actual cleavage sites.
Figure 4.5: pRSET b plasmid vector for the cloning of mCherry.

4.2.1. Variants of mCherry protein that conserve the chromophore after expression

From the two major classes of mCherry variants designed in Dr. Yang’s lab, the protein chromophore formation was examined during the expression by measuring red fluorescence using FluoStar. The only variants that form a wild-type chromophore throughout the protein expression are described in Table 4.1.

Table 4.1: Expression of mCherry variants that conserve the fluorescent chromophore

<table>
<thead>
<tr>
<th>Fluorescence</th>
<th>CaM Loop-I</th>
<th>CaM EF</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H17-D18</td>
<td>D115-G116</td>
<td>K56N</td>
</tr>
<tr>
<td></td>
<td>D115-G116</td>
<td>E153-G154</td>
<td>A145E, N196D, K198D, R216E (MC-1)</td>
</tr>
<tr>
<td></td>
<td>D169-G170</td>
<td>V187-Q188</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V187-Q188</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

mCherry protein variants grafted with loop-I and the complete EF hand motif-I of CaM at positions 17, 115, 153 169, 187 obtained significant increase in red fluorescence during expression of approximately 25 hours after culture transfer (Figure 4.5). The fluorescent intensity reached values ranging in the 200 and 470. These proteins gradually formed the chromophore after induction with 0.2mM IPTG. mCherry variant H17-D18 (MC-I-17) was expressed separately in the trials but this one exhibited the greatest fluorescent intensity after protein expression overnight. This mutant exhibited fluorescence up to 461. Direct mutations K56N, A145E, N196D,K198D, R216E (MC-1), and K198D,N196D,K198D,R216E (MC-2) also developed red fluorescence after expression with same IPTG addition (Appendix). The
expression of correct protein was monitored by SDS-PAGE. The SDS-PAGE shown in (Figure 4.6) suggested that the mCherry variants were expressed some even before induction with a 32KDa band.

Figure 4.5: Fluorescence intensity of mCherry variants inserted with loop-I and EF hand motif of CaM B. Cell pellets (c/p) were dissolved in 10mM Tris Buffer pH 7.4 and fluorescence measured a micro-plate reader using the FluoStar as instrument.
Figure 4.6: SDS electrophoresis showing the expression level of five E-I-F hand loop mutations in the mCherry peptide sequence. Molecular weight of mCherry is ~32KDa. Last gel shows protein expression for the mutation of His residue to Asp.

OD(600nm) expression of variants grafted with the EF hand motif I of calmodulin in mCherry at locations 115, 153, 169, and 187 were monitored every hour before induction, and 2, 4 and 25 hours after induction with IPTG. Cell growth was optimal after expression 25 hours reaching an OD (600) in the range 1.5 and 2.2. Protein expression was induced after 3 hours from inoculated transfer (see methods in chapter 2) when OD(600nm) reached 0.6 (Appendix). Wild type protein was used as control for both cell growth and protein expression.

4.2.2. **Fluorescent variants that DO NOT conserve the chromophore formation**

Table 4.2 summarizes the variants that do not have the chromophore formation after bacteria cell expression.
As compared with the proteins that developed chromophore formation, the only variants that form a wild-type chromophore throughout the protein expression are described in Table 4.2.

Table 4.2: Expression of mCherry variants that DO NOT conserve fluorescent the chromophore

<table>
<thead>
<tr>
<th>CaM Loop-I</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-color</td>
<td>F14-E15</td>
</tr>
<tr>
<td></td>
<td>G33-E34</td>
</tr>
<tr>
<td></td>
<td>A44-E45</td>
</tr>
<tr>
<td></td>
<td>E144-A145</td>
</tr>
<tr>
<td></td>
<td>Q42E</td>
</tr>
<tr>
<td></td>
<td>K80N</td>
</tr>
<tr>
<td></td>
<td>A217D</td>
</tr>
<tr>
<td></td>
<td>K144N</td>
</tr>
</tbody>
</table>

mCherry protein variants grafted with loop-I of CaM at positions 14, 33, 44, 144 did not obtain increase in red fluorescence after expression (Figure 4.5). The fluorescent intensity reached values ranging in the 35 and 55 that are meaningless when comparing with the WT that ranges in the 400.
Figure 4.7: Fluorescence intensity for the seven mutations of mCherry having mCherry wild type (WT) as control. Cell pellets (c/p) were mixed in 10mM Tris Buffer pH 7.4 in a micro-plate reader (FLUOSTAR). 1mL samples were collected during the expression of both proteins.

Cell growth was monitored by OD(600nm) before and after induction with IPTG. Overnight absorbance values were similar to the one for wild type approximately ranging from 2 to 5 with dilution of the sample in LB.

The SDS-PAGE in (Figure 4.8) shows some of the variants that did not form the chromophore after expression. It suggests that the proteins were expressed in accordance with cell growth shown by OD (600nm) although no fluorescence was observed.

![Figure 4.8: SDS electrophoresis gels showing the expression level of three of the new designed mutations in the mCherry peptide sequence. Molecular weight of mCherry is ~32KDa. 10uL sample added.](image)

Variants without formation of the red fluorescence chromophore presented almost clear bands with the molecular weight of mCherry (34KDa). Some variants were expressed before induction and others with higher intensity after induction overnight.

4.3. Purification of mCherry
4.3.1. Purification using a Histadine-Tag and affinity chromatography.

Protein purification of our calcium based sensors had been achieved using His-Tag affinity chromatography. mCherry variants containing the His-Tag can be isolated by the use of this
technique. (Figure 4.9) shows the SDS-PAGE for the fractions after elution of different protein variants of mCherry grafted with loop-I of CaM: 115-I, 169-I and 187-IA. The expression and purification of 145E N196D K198D R216E (MC-1) mutation with designed calcium binding pocket is observed, but this showed three bands with the molecular weights 32, 18, and 15 KDa respectively were detected after elution. The 32 band was expected, but 18 and 15 KDa were not and these are likely to be the cleavage products of two major fragments of the protein.

![SDS-PAGE purification of mCherry variants](image)

Figure 4.9: SDS-PAGE purification of mCherry variants with the insertion of loop I of calmodulin after elution using affinity chromatography His-Tag column. Last two pages show expression and purification for the calcium binding pocket E144, K198D, D200, Y214E, R216E mutated within mCherry.

### 4.4. Proteolytic analysis for mCherry mutation (MC-1)

An SDS-PAGE was run to test the sample buffer used for running the protein MC-1. Six samples of the protein mCherry WT were pre-treated with the addition of 20µL of sample buffer, some containing 5% β-mercapto-Ethanol with constant boiling for 5 minutes and without boiling
and the others containing addition of 5% β-mercapto-Ethanol again boiling and without boiling (Figure 4.10). The samples with β-mercapto-Ethanol with boiling showed three bands similar to the MC-1. Similar effect occurred for the boiled samples with no addition of β-mercapto-Ethanol. The samples that were not boiled developed a cleavage, but this one different from previous samples.

Figure 4.10: SDS electrophoresis gel showing the test done on 2-mercapto ethanol on sample buffer to run the gel page. Also, the samples were boiled and non-boiled for the analysis.

4.4.1. Sequencing Analysis of mCherry protein cleavage by the enzyme Trypsin /Chymotrypsin

Further analyses were performed to understand the cleavage on MC-1 and similar changes occurred in other mCherry variants. Sequence analysis was performed to predict potential cleavage sites comparing results from Mass Spec and SDS-PAGE. The protein MC-1 was used for analysis by Mass Spec (Figure 4.11). The three protein bands shown in SDS-PAGE were compared with the three peaks obtained from Mass Spec to predict the protease enzyme that was causing it.
Figure 4.11: Prediction of potential proteolytic Mass Spec data (MALDI) from MC-1 sample after purification with His-tag.

Mass Spec shows four major peaks. From right to left, the molecular weights represented by each peak are: 30.9, 18.7, 15.2, and 11.6 Kilo Daltons (KDa). We hypothesized that the proteases trypsin and/or chymotrypsin were causing the cleavage in two sites of mCherry on the outsider loops of the sequence as proposed in (Figure 4.12). Trypsin or chymotrypsin cut after the C-terminal of Lys (K) and Arg (R) or Phe (F), Tyr (Y), and Trp (W) respectively, except for residues preceding Pro (P). The molecular weight of the entire sequence of MC-1 and the possible cleaved sites were measured using the online aminoacid calculator at www.protincalculator.org. Molecular weights of cleaved sequences were compared to results from Mass Spec and SDS-PAGE. A summary of the work is shown in (Figure 4.13-4.14). All possible lysine (K) residues forming the loops of the sequence were mutated to Asparagine (N) (Figure 4.12).
Figure 4.12: Proposed binding sites for the protein mCherry binding to the affinity chromatography column (His-Tag).

Figure 4.12 shows that cleavage of mCherry (MC-1) determined by SDS-PAGE. First band at molecular weight 30.7KDa is the entire sequence of the protein, while bands at molecular weights of 23.8 and 15.9 KDa correspond to the cleaved sequences of the protein at two locations.

H17N S21E, K50N, K123L & R125L (No His-Tag)

<table>
<thead>
<tr>
<th>MW</th>
<th>E(280nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26170.2246</td>
<td>32430 M-1cm-1</td>
</tr>
</tbody>
</table>

MC-1

EEDNMAIKE FMRFKNMMEG NVNGHEFEIE GEGEGRTYEG TQTALKTVK GNPLPFPWDI 60
LSPQFMGSK AYYKRPADID DYLKLSPPEG FKVERNMNE DGGVVTQD SSLQDGEFYI 120
PULLGTMF SDGFMNVT MGEESER YPEDGALKGE IKQRLKLDG GH1DAEVKTH 180
YKAKKPVQLP GAYVNDIDLD ITSHEDYTI VEQVEEAEGR HSTGMDELY K 231

**MAD-1** (K15-N15) No Right sequence after PCR (Not needed MW= 6316Da)
**MAD-2** (K50-N50) Same as MC-1 mutation (not necessary)

**MAD-3** (K74-N74) Need to be expressed; MW= 12,788.2 (by sequencing) 3rd band

**MAD-4** (K138-N138) Need to be expressed; MW= 19,975.3 (by sequencing); 18,784 (Mass Spec)

**MAD-5** (F99-W99) Possible MW 15,818 Da (by sequencing); 15,237 Da (Mass Spec) 3rd band

Figure 4.13: Peptide sequence for the protein variant MC-1 (mCherry) showing the most possible cleavage sites by the enzyme trypsin. Underlined amino acids are loops of the peptide sequence of MC-1. Highlighted residues are the most possible sites for cleavage when compared to Mass Spec analysis.
From sequence analysis, we developed possible cleavage locations in MC-1 on the outside loops that were acting as points of action for the protease trypsin and/or chymotrypsin. Four main residues of the lysine residue K15, K50, K74, and K138 were picked to develop direct mutagenesis with asparagine (N). The 40 residues from His-Tag were not included for selecting the Lys mutations. Four new mCherry variants were designed using PCR. (Figure 4.14) shows the primer design of several variants for cloning.

Figure 4.13: Sequencing of the protein mCherry-1 (MC-1) with trypsin /chymotrypsin (Histadine-tag included). Two major cleavages occurred at residues K74 and K138. Mutations K50N (MAD-2), K74N (MAD-3) and K138N (MAD-4) were made to prevent cleavage

Four mutations were developed after PCR: MAD-1, MAD-2, MAD-3, and MAD-4 from mutations K15N, K50N, K74N, and K138N. MAD-3 and MAD-4 were of major focus since cleavage on these two sites would occur on three protein sequences with molecular weights:
30.5, 19.9, and 12.7 KDa. Primer designs for the mutations in lysines (K) on the main DNA sequence of MC-1 are described in (Figure 4.14).

Primer designs for the mutations in lysines (K) on the main DNA sequence of MC-1 are described in (Figure 4.14).

After obtaining the DNA for the four designed proteins: MAD-1, MAD-2, MAD-3, and MAD-4, we checked the protein sequences and only MAD-2, MAD-3, and MAD-4 were successfully mutated at the exact locations. These proteins were expressed in LB, but only MAD-2 developed red fluorescence after induction with IPTG. The expression was repeated and
results were similar. The protein mutation MAD-2 (K50N) was purified by His-Tag column and the cleavage still persists after elution.

4.5. Quantitative analysis of mCherry variants using UV-Vis absorbance and fluorescence spectroscopy

4.5.1. Measuring extinction coefficient of the chromophore of the mCherry-based sensor variants

In order to measure the extinction coefficient of the fluorescent protein mCherry, equation 4.2 was used. In (Figure 4.15 and 4.16) two protein variants of mCherry from class 1 (115-I and 115EF-I) were measured experimentally and compared with their reference mCherry WT at different concentrations to obtained the ratio at the wavelength of 586nm. Graphs for other grafting locations are in the Appendix.
Figure 4.15: A-B. Absorbance of mCherry WT and mCherry 115-I proteins reducing the protein concentration from 9.5µM to 0.5 µM. 10mM Tris buffer pH 7.4 was used for analysis. C-D. Ratio ABS (586nm) / ABS (280nm) from points from plots A and B respectively to determine Extinction coefficient.

Figure 4.16: A-B. Absorbance of mCherry WT and mCherry 115-EF proteins at different concentrations is described. 10mM Tris buffer pH 7.4 was used for analysis. C-D. Ratio ABS (586nm) / ABS (280nm) from points from plots A and B to determine Extinction coefficient.

The Figures 4.15 and 4.16 show plots of absorbance at different protein concentrations to compare the chromophore excitation when this absorbs light at 590nm. Sample proteins were diluted from 9.5 µM to 0.5 µM to plot the values of absorbance at 586nm as a function of absorbance at 280nm. By fitting the equation, the extinction coefficient can be obtained from the slope of the line. The extinction coefficient of the protein variants mCherry 115-I and 115-EF-I can be obtained by comparing it with the one obtained for the protein wild type. A summary of calculations to find the extinction coefficient from the previous analysis is shown in Table 4.3.
Table 4.3: Mathematical representation to determine molar extinction coefficient is explained for two engineered variants of mCherry. Slope obtained using the ratio from points ABS (586) vs. ABS (280) is used for the analysis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Slope</th>
<th>$\varepsilon$ (280nm)</th>
<th>$\varepsilon$ p(586nm) Experimental (mM$^{-1}$cm$^{-1}$)</th>
<th>$\varepsilon$ p(586nm) modified (mM$^{-1}$cm$^{-1}$)</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equation</td>
<td></td>
<td>32,890mM$^{-1}$cm$^{-1}$</td>
<td>$\left(\frac{A_p\text{, }586\text{nm}}{A_p\text{, }280\text{nm}}\right)\varepsilon_p(280\text{nm})$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mCherry WT</td>
<td>1.16</td>
<td>32,890mM$^{-1}$cm$^{-1}$</td>
<td>22,286</td>
<td>72,000</td>
<td>100%</td>
</tr>
<tr>
<td>mCherry 115-I</td>
<td>1.24</td>
<td>32,890mM$^{-1}$cm$^{-1}$</td>
<td>24,154</td>
<td>78,035</td>
<td>108%</td>
</tr>
<tr>
<td>mCherry 115 E-I-F</td>
<td>0.73</td>
<td>32,890mM$^{-1}$cm$^{-1}$</td>
<td>19,129</td>
<td>61,803</td>
<td>85.8%</td>
</tr>
</tbody>
</table>

The table shows the calculations to obtain the extinction coefficient of the protein variants. The product of the experimental extinction coefficient obtained from the slopes (Figures 4.15 and 4.16) and the extinction coefficient at 280nm from sequencing the protein residues using a protein calculator is equal to the final extinction coefficient at 586nm for the protein variant. This final value is modified by comparing it with its wild type that has been calculated in other literature.

4.5.2. Measuring Quantum yield and brightness of mCherry calcium-based sensor variants

In order to measure the quantum yield abbreviated by the symbol ($\Phi$), it is necessary to use equation 4.3. In the graphs below (Figure 4.17 and 4.18), a similar analysis as the one done for determining the extinction coefficient is performed for quantum yield ($\Phi$). Same samples were used to measure fluorescence at emission 608nm. The same principle as before is applied to obtain the values using the equation.
Figure 4.17: A-B. Fluorescence of mCherry WT and mCherry 115-I proteins at different pH is described. 10mM Tris buffer at different pH were used for analysis. C-D. Ratio Fluo (608nm) / ABS (586nm) from points from plots A and B to determine quantum yield.
Figure 4.18: A-B. Fluorescence of mCherry WT and mCherry 115-E-I-F proteins diluted to different concentrations is described. 10mM Tris buffer pH 7.4 was used for analysis. C-D. Ratio Fluo (608nm) / ABS (586nm) from points from plots A and B to determine quantum yield.

Protein variants 115-I and 115-EF-I were again measured to determine their quantum yield of fluorescence. Sample proteins were diluted from 9.5 µM to 0.5 µM to plot the values of fluorescence at 608nm as a function of absorbance at 586nm. By fitting the equation, the quantum yield can be obtained from the slope of the line. The quantum yield of the protein variants mCherry 115-I and 115-EF-I can be obtained by comparing it with the one obtained for the protein wild type. A summary of calculations to find the quantum yield from the previous analysis is shown in Table 4.4.

Table 4.4: Mathematical representation to determine quantum yield.
4.5.3. Measuring pKa of mCherry calcium-based sensor variants

pKa values were obtained by measuring absorbance of the proteins at different pH values. Starting from the most stable protein pH and continuously going down in a one unit scale and then 0.5 scale when approaching the equilibrium point. pH Tris buffer using a whole set of buffers with different pH values. These pKa values can be obtained by the fitting absorbance/pH change explained in the equation 4.5. The pKa values only for the protein mCherry variants grafted with the calcium binding loop-I of calmodulin have been obtained (Appendix). The absorbance graphs to determine pKa for the mutant mCherry 115-I and its control mCherry WT are shown in (Figure 4.19).
Figure 4.19: A-B. Absorbance of mCherry WT and mCherry 115-I proteins at different pH values is described. C-D. Titration plot using absorbance intensity at 586nm as a function of pH to find pKa = m3.
pKa values were obtained using buffers at different pH values. The protein variants were titrated using the same concentration. A plot was constructed from both absorbance at 586nm and fluorescence at 608nm. The pKa value for each variant was obtained fitting the points of titration using the equation 4.3. The m3 value represented the pKa of the protein.
The analysis of 8 mCherry variants with the red fluorescence unperturbed after expression was established. Table 4.5 gives a summary of this analysis with their control mCherry WT.

Table 4.5: Spectroscopic and chemical properties of mCherry WT and its counter variants grafted with the loop-I and EF hand motif. Calcium binding sequences of the protein calmodulin (CaM).

<table>
<thead>
<tr>
<th>Protein</th>
<th>ε (mM⁻¹ cm⁻¹)</th>
<th>Φ</th>
<th>Brightness (ε)ₓ(Φ)</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCherry WT (control)</td>
<td>72</td>
<td>0.22</td>
<td>15.8</td>
<td>100.0</td>
</tr>
<tr>
<td>mCherry-I-115</td>
<td>78.0</td>
<td>0.24</td>
<td>18.7</td>
<td>118.4</td>
</tr>
<tr>
<td>mCherry-I-153</td>
<td>56.5</td>
<td>0.22</td>
<td>12.6</td>
<td>79.8</td>
</tr>
<tr>
<td>mCherry-I-169</td>
<td>62.8</td>
<td>0.22</td>
<td>14.1</td>
<td>89.2</td>
</tr>
<tr>
<td>mCherry-I-187</td>
<td>37.9</td>
<td>0.21</td>
<td>8</td>
<td>50.8</td>
</tr>
<tr>
<td>mCherry-EIF-115</td>
<td>61.8</td>
<td>0.29±0.06</td>
<td>17.9</td>
<td>123.9</td>
</tr>
<tr>
<td>mCherry-EIF-153</td>
<td>117.5</td>
<td>0.24±0.06</td>
<td>28.2</td>
<td>178.5</td>
</tr>
<tr>
<td>mCherry-EIF-169</td>
<td>93.3</td>
<td>0.22±0.06</td>
<td>20.5</td>
<td>129.7</td>
</tr>
<tr>
<td>mCherry-EIF-187</td>
<td>89.0</td>
<td>0.22±0.06</td>
<td>19.6</td>
<td>124.6</td>
</tr>
</tbody>
</table>

4.6. Conclusions

In this study, 21 mCherry-based calcium sensors have been designed and engineered, which have been well expressed in *E. coli*. Only 12 variants maintained red fluorescence after expression overnight. Eight of these mCherry-based calcium sensors grafted with loop-I and EF hand motif I of CaM at residue 115, 153, 169 and 187 exhibited wild type like chromophore formation, extinction coefficient, and quantum yield (Table 4.5). The pKa of the chromophore was not altered after modification. Grafting loop-I of CaM at position Q42-E43, A217-D218, G33-E34, A44-E45, F14-E15 and E144-A145 resulted in significant loss of fluorescent intensity.
while proteins were expressed. Table 4.5 shows literature samples and from these values, we are now able to compare data from other researchers and publish our results. Our engineered mCherry variants exhibit a greater chromophore extinction coefficient than the reported values from Campbell et al in (Table 4.6). These variants have a small loop inserted into 22, 26, 38, 84, and 93 positions of mCherry and their brightness, which is the product of extinction coefficient and quantum yield in proteins, is reduced. On the other hand, our insertion of EF-I to 115, 153, 169, and 187 do not reduce optical properties.

Two mCherry-based designed calcium sensors (MC-1, MC-2) have been also expressed and purified. These two mutations from class 2 have been able to bind calcium and also exhibit calcium-induced fluorescence change (Appendix). WT mCherry and its variants exhibited cleavage fragments in the range of 31, 24, and 16 KDa after purification by His-Tag binding affinity chromatography. Detailed sequence analysis for potential proteolytic cleavage was performed. Four potential locations K15, K50, K74 and K138 were likely the cleavage sites especially the last two since they produced fragments with observed molecular weight in that range. We then cloned four variants K15N, K50N, K74N and K138N. Single mutation at K74N and K138N eliminated chromophore formation, indicating that mutated lysines (K) at these positions are essential for chromophore formation and protein maturation. K50N developed the chromophore, but cleavage fragments still were present after purification. Grafted sequences of mCherry with loop-I of CaM produced a small response for calcium binding, while the calcium-binding capability of calcium sensors with grafted EF-hand motif need to be studied.

Table 4.6: Properties of literature mCherry variants.
CHAPTER 5
5. X-RAY CRYSTALLOGRAPHY STUDY ON THE GREEN FLUORESCENT CALCIUM SENSORS: D11 AND G1

5.1. Introduction

5.1.1. Properties of Green Fluorescent Proteins (GFP)

Green Fluorescent Protein (GFP) has become one of the most widely studied and exploited proteins in biochemistry. It was discovered in 1962 by Shimomura et al in the jellyfish Aequorea victoria [50]. GFP’s amazing ability to generate a highly visible, efficiently emitting internal fluorophore without a substrate is both intrinsically fascinating and tremendously valuable by many researchers. The independent intrinsic fluorescence is due to the formation of its chromophore, which occurs spontaneously in the middle of the β-barrel through a cyclization process involving residues S65, T66, and G67 [51]. Interestingly, this process is temperature dependent, and GFP must be expressed at lower temperatures (below 30 °C), to ensure proper formation of the chromophore. This is likely due to the fact that A. Victoria lives in cold water. GFP has become well established as a marker of gene expression and protein targeting in intact cells and organisms. The fluorescent properties of GFP have led to its use in a number of different applications. The first proposed application of GFP was the detection of gene expression in vivo by Chalfie [52]. It has also been utilized as both active and passive reporters of cell function in molecular and cell biology, and used as a gene marker, which can be easily detected by fluorescence microscopy or flow cytometry. Viable GFP-positive cells have been isolated through fluorescence-activated cell sorting. Through these creative applications, GFP has become as a versatile tool for the study of gene expression, gene transfer, protein folding and trafficking, and protein-protein interactions due to its spectral characteristics, small size, and acquisition of fluorescent activity in absence of any other cofactors. In vivo, applications of GFP
can be divided into two categories based on its use, as either a tag or an indicator. As a tag, GFP fluorescence reflects levels of gene expression or sub-cellular localizations of the host proteins to which GFP is fused. As an indicator, GFP fluorescence is modulated post-translationally by its chemical environment and protein-protein interactions[51]. The first proposed application of GFP was the detection of gene expression in vivo by Chalfie[52].

Currently, GFP is used for determining various phenomena in various compartments of cells, tissues, or organs (Figure 5.1), so it has figured prominently in our research to develop fluorescent calcium sensors, acting as a scaffold for protein engineering whereby a Ca^{2+}-binding motif is grafted into the protein in close proximity to the natural chromophore in order to exploit internal fluorescent changes observed during Ca^{2+}-binding in vivo.

![Figure 5.1: Fluorescent proteins have been very useful for watching invisible processes: proteins allow the monitoring in time and space of a big number of phenomena in living cells.](image)

5.1.2. **Protein crystallography study**

Protein crystals are precisely ordered three-dimensional arrays of molecules that may be characterized by a concise set of determinants. These determinants exactly define the disposition and periodicity of the fundamental crystal units of which these are composed[53]. The final successful result generates a 3D array of periodically repeating unit cells that can be translated
via interpretation of X-ray scattering into a protein crystal structure. Some examples of GFP crystals are shown in (Figure 5.2a). Characteristics of crystals, unit cells and Bravais lattices are illustrated in (Figure 5.2b). Macromolecular crystallization is a process that requires individual systematic analyses of parameters that create the best environment for crystal formation; identifying one or more sets of these factors that yield crystals, and then optimizing the variables to obtain the best possible crystals for X-ray analysis [54]. This is established by conducting a vast array of crystallization trials, evaluating the results, and using information obtained to improve matters in successive rounds of trials. Intelligence and intuition becomes the most essential tool for evaluating and designing these trials [54].

Figure 5.2a: Photographs of GFP. A. Hexagonal crystals. B. Monoclinic parallelepipeds. The bar in the lower corner represents 0.5mm.
Figure 5.2b: The 14 types of unit cells that form the basis for the allowable lattices of all crystals (known as Bravais lattices). All primitive (P) cells may be considered to contain a single lattice point (one-eighth of a point contributed by each of those at the corners of the cell), side-centered and body centered (bcc) cells contain two full points, and face centered (fcc) cells contain four complete lattice points.

In order to study our calcium protein sensors by X-ray crystallography, there is a sequence of crystallization steps involved starting from crystal growth to the protein structure study (Figure 5.3). When these sensors are successfully crystallized, a door will open to improve our studies and come out with better designs for calcium binding modeling.
5.1.3. Why crystals grow

Systems always tend to proceed to equilibrium. By doing this Entropy (extent of disorder) increases. There is a thermodynamic tendency to minimize Gibbs free energy of the system. This is achieved by the formation of chemical bonds and interactions that provide the negative transition. The assembly of molecules into a fixed lattice reduces mobility and freedom, yet crystals form and grow. Gibbs free energy is defined by equation 5.1.

\[
G(p, T) = U + pV - TS \quad \text{Increase in } S
\]

Or

\[
G(p, T) = H - TS \quad \text{decrease in } G
\]

\[
\Delta G = \Delta H - T\Delta S
\]

Equation 5.1
Entropy increases because the system frees individual constituents from physical and chemical constraints. These bonds are in fact what hold crystals together. To enhance crystal growth, then it is necessary to ensure the greatest number of most stable interactions among the molecules in the solid state.

5.1.4. **Drawbacks in crystallization**

Crystallization requires the gradual creation of a supersaturated solution of increasingly larger molecules. As temperature, pH, Pressure and solvation are changed, so the conformation, charge state, and size of the macromolecule change. Large molecules are very sensitive. They can denature or degrade in many ways. They must be maintained in a thoroughly condition throughout growth.

5.1.5. **X-Ray diffraction for analysis**

X-Rays are a form of electromagnetic radiation that have wavelengths in the range of 10 to 0.01nm and energies in the range 120 eV to 120 keV. X-rays are produced when electrons jump from a higher energy state to a lower one (Figure 5.4).
Figure 5.4: X-rays have a wavelength in the range of 10 to 0.01 nm and energies in the range 120 eV to 120 keV.

About 95% of all solid materials can be described as crystalline. When X-rays interact with a crystalline substance (Phase), one gets a diffraction pattern (Figure 5.5). The X-ray diffraction pattern of a pure substance is, therefore, like a fingerprint of the substance. The powder diffraction method is thus ideally suited for characterization and identification of polycrystalline phases. The two parallel incident rays \( k_i \) and \( k_f \) form an angle \( \alpha \) with these planes. A reflected beam of maximum intensity will result if the waves reflected are in phase.

Figure 5.5: X-ray diffraction sketch from analysis of a protein crystal.

The atoms are arranged in a regular pattern, and there is as smallest volume element that by repetition in three dimensions describes the crystal. This is analogous to describing a brick wall by the shape and orientation of a single brick. This smallest volume element is called a unit cell. The dimensions of the unit cell are described by three axes: a, b, c and the angles between them \( \alpha, \beta, \gamma \) (Figure 5.2).
5.1.6. Developed calcium sensors
5.1.7. Objectives of this research

Dr. Jenny Yang’s lab has created two GFP calcium sensors from the Green Fluorescent Protein (GFP): EGFP-7E15 or D11 and EGFP 172EF-III or G1 (Figure 5.6). Both sensors have calcium binding abilities while these were tested with different calcium concentrations, we can see the increase in fluorescent intensity by adding addition of calcium (Figure 5.6). The protein sensor 7E15 (D11) was created by the graduate student Ada Tang by designing a calcium site into GFP. This sensor is able to exhibit fluorescence signal at 509 nm upon calcium binding when excited at 488 nm. The second sensor EGFP172 EF-III (G1) was created by grafting the EF hand motif (III) of CaM into position 172 by Dr. Jin Zou. This sensor, exhibits a ratiometric fluorescence change when excited at 398 and 490 nm; fluorescent signal increases and decreases upon the binding of calcium. One of the best ways to determine structures of designed calcium sensors is by x-ray crystallography analysis. Protein crystallography will help us to determine the coordinations of designed calcium binding proteins. Determination of structure by X-ray is also advantageous especially for large protein complexes and speed. The significance for obtaining structural information of designed calcium sensors is several folds. First, it allows us to understand key factors for chromophore change upon calcium binding. Second, we can determine coordination of created calcium binding sites. Third, we can obtain information chart of calcium induced conformational change. Fourth, all this information will facilitate future designs for calcium binding sites. The main focus of this chapter is to identify optimal conditions for growing protein crystals from the engineered based calcium sensors of GFP (D11 and G1). Each protein sensor is different in structure and screening the best buffer conditions has come to be our major challenge in this project.
5.2. Expression and purification of GFP calcium sensors

From the methods described in Chapter 2, we were able to express and purify mutations of GFP with the insertion of the calcium binding motif EF hand motif of CaM. The expression of two sensors D11 and G1, appear next to the wild type (WT) control (Figure 5.7).
Figure 5.7: OD (600nm) to monitor cell growth of the cell strain BL21-DE3 from *E.coli*. Two calcium sensors of GFP designed by Ada Tang 7E15 (D11) and Dr. Jin Zou 172EF-III (G1) are expressed. GFP WT was used as a control. Culture samples were grown 14-17 hours after the induction with IPTG.

To purify the EGFP calcium based sensors, cell pellets were re-suspended in 10 ml of lysis buffer and sonicated to disrupt the cell membrane. The solution was centrifuged at 17000 RPM for 20 min, and the supernatant was filtered and injected into a nickel-chelating column loaded with 0.1 M nickel sulfate solution on fast performance liquid chromatography (FPLC) as described in chapter 2. After washing with buffer A (50 mM phosphate, 250 mM NaCl, 40 pH 7.4), the bound protein was eluted with a gradient of imidazole from 0 to 0.5 M in phosphate buffer (see methods). The purity of the fractions was monitored by SDS-PAGE (Figure 5.8). The protein collected from FPLC was dialyzed in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4) to remove imidazole. The concentration of purified protein was determined by UV-visible absorbance at 280 nm with an extinction coefficient constant of 21,890 M$^{-1}$ cm$^{-1}$. The concentration after binding affinity came to be 46.1 μM (18mL) (Appendix). After concentrating down the 3 (6mL) eluted fractions, 2.25mL of 369.1μM (12.2mg/mL) protein was obtained.

The SDS-PAGE in (Figure 5.8) reveals the protein expression at various stages during cell growth and purity after binding affinity and gel filtration. At the end the protein obtained contained a high yield good for protein crystallography analysis.
5.3. **Purification of EGFP using gel filtration**

For further purification, the protein is concentrated down to 12.2 mg/mL (369.1 μM). Any green fluorescent precipitate is re-dissolved with 10 mM Tris buffer pH 7.4 same as the one used during protein dialysis. The protein is then purified by Fast Pressure Liquid Chromatography (AKTA prime) with a 120 mL gel filtration column (Superdex 75) using manual operation and Tris buffer. Ultraviolet/visible absorbance is performed from 200 to 500 nm on a Beckman UV-1601 with deuterium/tungsten lamps on FPLC fractions to determine concentration (Figure 5.8).

5.4. **Growing crystals from GFP mutants**

Proteins have been crystallized for many different purposes: a structural model of GFP based on a single crystal x-ray diffraction analysis is crucial to the resolution of a number of questions concerning the nature of the energy transfer mechanism [12]. Also, the notable stability of the molecule and details of the structure and environment of the unique chromophore are of real importance in research. The complementary structural data obtained from fluorescence spectroscopy and x-ray diffraction crystallography will aid in the elucidation of novel protein’s
structure-function relationship. In the past, the energy transfer protein, green fluorescent protein (GFP), from the hydromedusan jellyfish *Aequorea Victoria* was crystallized in two morphologies suitable for x-ray diffraction and then structure analysis. The most striking feature of the protein is its bright green fluorescence, which has been extensively studied in many works [55].

To separate isoproteins such as the three Ca$^{2+}$ sensors of GFP (WT, D11, and G1) in our lab, samples were purified using binding affinity chromatography and gel filtration chromatography. According to Perozzo, GFP crystals grow in 4-7 days and their growth rate and crystal size depended on purification effort. Average crystal dimensions commonly found have been close to these: 0.1 x 0.1 x 0.8 mm for the monoclinic parallelepipeds and 0.4 x 0.4 x 0.1 mm for the hexagonal plates.

5.4.1. **Screening and optimization for crystal growth of GFP Ca$^{2+}$ based sensor (7E15 or D11)**

There are basically two approaches to screening for crystallization conditions. The first is a systematic variation of what it is believed the most important variables, precipitant type and concentration, pH, temperature and so forth. An example of this strategy is shown in (Figure 5.9), in which the screening changing pH and PEG concentration is first developed to match the best conditions for the protein; the best range is optimized and more carefully analyzed. The second is what we might consider a shotgun approach, but a shotgun aimed with intelligence, experience and accumulated wisdom [54].
All buffers must be filtered before use.

<table>
<thead>
<tr>
<th>Protein Variant</th>
<th>HEPES (mM)</th>
<th>PEG 4000</th>
<th>MgCl₂ (mM)</th>
<th>B-Mercapto Ethanol (mM)</th>
<th>pH</th>
<th>Protein (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7E15 (Apo) D11</td>
<td>50</td>
<td>13%</td>
<td>50</td>
<td>1</td>
<td>6.8</td>
<td>360 13 mg/mL</td>
</tr>
</tbody>
</table>

Figure 5.9: Crystal screening for the Ca²⁺ based sensor protein EGFP (7E15) changing pH (6.5-8.0) and PEG 4000 (10-15%).

Best screening of D11 in the Apo (Ca²⁺ unbounded) form occurs at pH 6.8 and PEG (4000) 13%. A well semi-monoclinic crystal grows as pictured in the figure. More crystals were obtained using the same conditions. Crystals of the protein D11 (Apo form) normally take 4-5 days to develop under these conditions.

### 5.4.2. GFP calcium sensors crystallized

Throughout different conditions for crystal screening of GFP 7E15 and GFP 172EF-III (Figure 5.10), we were able to approach the best pH and PEG conditions to grow crystals of different magnitudes and shapes. Table 5.1 provides the best screening conditions for our protein sensors crystallized in our laboratory. Sample crystals in the Apo (calcium unbounded: left) and Holo (calcium bounded: middle) forms were both crystallized using similar buffer conditions.
(Figure 5.11). The protein G1 in the (Apo form) developed crystals, but a better screening needs to be designed since micro-crystals appeared to be overlapping each other, forming crystal clusters. These clusters can be avoided by reducing crystallization temperature in order to reduce nucleation process. In general, in order to crystallize proteins, one needs to determine the precipitation points of the protein at sequential pH values with a given precipitant, repeat the procedure at different temperatures, and then examine the effects of different precipitating agents. Currently, there are numbers of devices and methods for bringing about the supersaturation of a protein solution, generally by the increase in concentration of some precipitant such as salt or poly(ethylene glycol) PEG. The well known salting-in method for modification of pH, salts, and ligands that we used to alter protein solubility is really common in many procedures of crystal growth.

Figure 5.10: Crystals obtained from the screening method developed in the lab varying PEG (3350/4000) concentrations and buffer pH at 25°C.
Table 5.1: Optimization of conditions for growing GFP 7E15 (D11) and 172 EF-III (G1).

<table>
<thead>
<tr>
<th>Protein Variant</th>
<th>HEPES (mM)</th>
<th>PEG 3350 or 4000</th>
<th>MgCl$_2$ (mM)</th>
<th>B-Mercapto Ethanol (mM)</th>
<th>pH</th>
<th>Protein (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7E15 (Apo) D11</td>
<td>50</td>
<td>13% (4000)</td>
<td>50</td>
<td>1</td>
<td>7.0</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13 mg/mL</td>
</tr>
<tr>
<td>7E15 (Holo) D11-Ca$^{2+}$</td>
<td>50</td>
<td>15% (3350)</td>
<td>50</td>
<td>1</td>
<td>7.0</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13 mg/mL</td>
</tr>
<tr>
<td>172 EF (Apo) G1</td>
<td>50</td>
<td>13% (4000)</td>
<td>50</td>
<td>1</td>
<td>6.8</td>
<td>540</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17.8 mg/mL</td>
</tr>
</tbody>
</table>

5.4.3. Mounting Crystals into liquid nitrogen

Mounting protein crystals into liquid nitrogen can be achieved by preparing a gradient with the mother buffer or same conditions the crystal grew with. This buffer is prepared with different glycerol percents (depending on protein) to maintain crystal stability during the transfer (Figure 5.11).
GFP crystals grew in the range of 0.1 and 0.2 mm and most maintained lattice structure after being in the micro-plate for two months. We were able to collect the crystals using a 0.1-0.3mm loop. In order to do this, it is required to open the sealed cover slip really gently and in a fast manner, transfer the crystal to the parker or crystal holder and store it into liquid nitrogen. Note: the crystal should not be exposed to an extensive period of time (more than 2 minutes) under the microscope light after opening the slip cover. The drop will evaporate and the crystal will start degrading. When the crystals are stored into liquid nitrogen, they will be sent to Argonne, Chicago for diffraction analysis.

6. **Conclusions and significance**

Two calcium sensors GFP (7E15 and GFP172) have been well expressed and purified using His-Tag affinity column. Gel filtration has applied to improve the protein purity required for
crystal growth. Protein concentration was reached to 13mg/mL from 1L cell pellet protein expression. Based on published crystallization condition of GFP (Temperature, pH, and PEG salt concentration), a new screening condition was designed and our GFP calcium sensors were well crystallized in 2-5 days under best suitable conditions. Buffer conditions for crystal growth have been screened for both calcium-free (Apo) and calcium-loaded (Holo) forms of both calcium sensors. The optimal crystallization conditions described in table 5.1 make crystal to grow 0.1-0.2mm in size. The green color and softness of crystals confirmed a high quality for X-ray diffraction analysis.

As a general case, in any protein crystallography study, it is important to purify the protein of study in order to avoid factors that would inhibit crystal growth. These factors obtained in non-homogeneous proteins will impose a generally negative effect on the attachment rate. The accumulations of contaminants will produce defects, dislocations and probably termination of crystal growth. For the crystal growth of our GFP sensors, it was advised that His-Tag binding affinity column and gel filtration column were used to obtain high quality of crystals.
6.0. Final conclusions:

Calcium is a fundamental element for living organisms in earth. It regulates a great number of biological and cellular signaling pathways. In cells, calcium binding proteins provide a major role in the transport of these ions to specific locations using their different metal binding capabilities. Based on metal charge, concentrations, and environmental conditions these ions manage to go from place to place using metal-binding proteins such as calmodulin (CaM) and calsequestrin (CSQ). These proteins have the ability to undergo different conformational changes upon the metal binding. These protein changes occurring within the cell create major cell responses including: the triggering of proteins, cell cycle and its death, the buffering of proteins, gene expression, and enzyme activation among others. Chemical and biological investigations have been designed to understand the role of calcium using engineered calcium binding proteins. The fundamental objective of this thesis was to monitor protein expression and to optimize purification on developed calcium engineered proteins to finally analyze them by quantitative analysis and crystallography studies.

In Dr. Jenny Yang’s lab, three approaches have been developed to understand these major processes. The first one is the design approach, which focuses on the prediction of Ca$^{+2}$ binding sites using computer algorithms, which provide rational basis for grafting them into host proteins such as GFP or mCherry proteins. The second is the grafting approach; this one is used to engineer calcium binding sites into scaffold host fluorescent proteins. The third is called the subdomain approach, which allows the study of the interaction between calcium binding sites, their conformational changes and cooperative interaction upon its binding. In this thesis, I introduced the three classes of proteins for calcium studies: 1) the grafted mCherry protein
variants with the calcium binding EF hand motif of calmodulin, 2) the mutated mCherry proteins to develop new calcium binding sites and 3) the mutated GFP to develop calcium binding sites. My work has been focused in reporting fluorescence changes while expressing new mCherry and GFP variants, analyzing the best conditions for protein purification, and protein studies such as the one developed for mCherry variants in determining the molar extinction coefficient, quantum yield and pKa, and GFP calcium based sensors in determining best crystallography conditions.

In chapter 3, we developed a study on bacterial cell growth and protein expression. We were able to identify how calcium and pH affect cell maturation and therefore protein expression. The major conclusions obtained were the following: 1) Extra cellular calcium (10mM) concentrations do not play a major effect in bacteria cell growth using the cell strain of E. coli BL-21(DE3), but increases protein expression after induction with IPTG overnight (15%) comparing it with the negative control (LB only); 2) pH affects both cell growth and protein expression. A 37% and 90% decrease respectively was obtained comparing to the control wild type.

In Chapter 4, the main characteristics in protein expression, purification and quantitative analysis of major variants of mCherry were obtained. This work required the generation of capable binding calcium sensors that kept normal characteristics after the insertion of grafting subdomains to the host protein. Many mCherry protein mutations with His-Tag were expressed to develop good calcium sensors that will bring great significance for research. Many of these proteins were successfully expressed and purified as fusion proteins with pRSETb vector transformed into E-coli cell strain BL-21(DE3) using IPTG as inducer. The protein variants that clearly showed a high fluorescent intensity were taken for purification using His-Tag binding affinity chromatography using Ni$^{2+}$ as the metal binding ions. The purification results for these proteins showed concentrations ranging from 50 to 150 μM in a ~6mL volume (2-5mg/mL),
which were enough to perform different studies. The fluorescent protein mCherry is of major significance for research due to the optimal fluorescence intensity, longer excitation and emission wavelengths, which lowers cell photo-toxicity, and low photo-bleaching level. The only limitation of mCherry is that the sequence is easily cleaved after expression and purification. From the work done, there has not been a major improvement for preventing it.

In chapter 5, I discussed the best conditions to grow crystals for the GFP based calcium sensors 7E15 (D11) and 172EF-III (G1). Both mutations showed high affinity to calcium after analysis. Three-dimensional structures for these calcium sensors will be of major significance for our research since we will be able to understand how designed mutations and grafted sequences within GFP work in metal binding and how these increase chromophore fluorescence. Although, we have not diffracted our grown crystals yet, they have fulfilled all degrees of purity and structure lattice necessary for X-ray diffraction. To screening for crystallization conditions, we needed to consider every component in the solution yielding crystals (buffer, salt, ions, etc), along with pH temperature, and the purity of the protein might have an impact on the quality of results. Crystallization of a novel protein using any method is unpredictable and we experienced several problems in this crystallization process such as protein precipitations, purity level, and protein loss. Overall, every macromolecule is unique in its physical and chemical properties due to the fact that amino acid sequences produce an unique 3D structure having distinctive surface characteristics.
References

11. Dirksen WP, L.V.e.a., *A mutation in calsequestrin, CASQ2D307H, impairs Sarcoplasmic Reticulum Ca2+ handling and causes complex ventricular arrhythmias in mice*. Department of Physiology and Cell Biology, 304 Hamilton Hall, 1645 Neil Ave, The Ohio State University College of Medicine, Columbus, OH 43210, USA.
44. Dennis, A.M.B., Gang. Fluorescence resonance energy transfer between a fluorescent protein and commercially available quantum dots.
APPENDIX A

Recipes and protocols:

**LB (For 1 Liter)**

<table>
<thead>
<tr>
<th>Component</th>
<th>liquid</th>
<th>plates</th>
<th>top agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
<td>10 g</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5 g</td>
<td>5 g</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
<td>10 g</td>
<td>10 g</td>
</tr>
<tr>
<td>Agar</td>
<td>-</td>
<td>15 g</td>
<td>7 g</td>
</tr>
</tbody>
</table>

1. Combine the tryptone, yeast extract, and NaCl with 950 ml of deionized water. Mix the solution until dissolved.
2. Adjust the pH to 7.0 with 5 N NaOH (will take about 0.2 ml). If making solid media (for plates or top agar) add the appropriate amount of agar after adjusting the pH.
3. Adjust volume to 1 liter with water.
4. Sterilize by autoclaving.
5. After autoclaving add antibiotic, if desired. Add chloramphenicol to a final concentration of 10 µg/ml and ampicillin to a final concentration of 50 µg/ml.

**Antibiotics**

**Ampicillin:**
Prepare a stock solution of 100 mg/ml in deionized water and filter sterilize it with a 0.45 µm filter. To prepare selective medium, cool medium to ~50°C after autoclaving, and add 1 mL of the ampicillin stock per liter of media (both liquid and solid) for a final concentration of 100 mg/ml. Store the stock solution at -20°C.

**Kanamycin:**
Prepare a stock solution of 30 mg/ml in deionized water and filter sterilize it with a 0.45 µm filter. To prepare selective medium, cool medium to ~50°C after autoclaving, and add 600 µL of the kanamycin stock per liter of media (both liquid and solid) for a final concentration of 50 µg/ml. Store the stock solution at -20°C.

**100 mM IPTG** For 10 ml of a 100 mM solution:
Dissolve 0.24 g of IPTG in sterile, deionized water. Bring the final volume to 10 ml and filter sterilize (0.22 µm filter). Do not autoclave.

50 mM CaCl$_2$ For 100 ml of a 50 mM solution:
Dissolve 0.56 g of anhydrous CaCl$_2$ (MW = 111) in 100 ml of deionized water. Filter sterilize (0.22 µm filter) or autoclave. Use this solution ice cold for competent cell preparation.

2x SDS sample buffer
For 10 ml, combine 2.5 ml of 0.5 M Tris-HCl, pH 6.8, 0.4 g SDS, 2 ml glycerol, 0.2 ml β-mercaptoethanol or dithiothreitol (DTT), and 0.1 mg bromophenol blue. Bring up the volume to 10 ml with deionized water and mix. Store in 1 ml aliquots at -70°C.

Buffers for His tag purification:

Extraction buffer: (for 1 L)

- 20 mM Tris (pH 8.0) 2.42 g
- 100 mM NaCl 5.84 g
- 0.1 % Triton X-100 1 ml of stock

Nickel-chelating column Buffer A: (**filter before use**) (for 1 L)

- 50 mM PO$_4$ (pH 7.4) 200 ml of 250 mM
- 250 mM NaCl 14.61 g

Nickel-chelating column Buffer B: (**filter before use**)(for 1 L)

PO$_4$ Buffer A with 0.5 M imidazole 34.04 g
(1,2-Diaza-2,4-cyclopentadiene) F.W. 68.08 g/mol

Protein Crystallography Documentation:

Expression and Purification of GFP 7E15 and 172 EF-III (D11 and G1):
GFP 172 was expressed and purified using binding affinity (Histag), further purification is required with gel filtration was done to get high protein concentration. For Expression of GFP 172, LB media was made with 25g LB broth dissolved in 1L dH2O in a 2L flask and autoclaved at 121°C for 45 minutes. A single culture was inoculated overnight at 37°C in 10mL of LB with 50 µg/mL kanamycin (6µL from stock solution 30mg/mL). Inoculum was added to previously made LB-Kan for a 1/100 dilution. Cells were allowed to grow in a shaker at 37 °C until the OD<sub>600</sub> reached 0.6. The growth was then induced with 0.2mM Isopropyl-β-D-thiogalactopyranoside (IPTG) and allowed to grow for 24 hrs at 25 °C. The growth was spun in Oak Ridge tubes at 7 K for 20 mins at 4 °C. The supernatant was poured off, and the cell pellets were scraped into a 50 mL Falcon tube. The cell pellet was stored at –20 °C for purification. A cell growth curve is then constructed, using the O.D. readings, and an SDS-PAGE gel is run for all aliquots taken during expression to determine if the desired protein was expressed. In addition, fluorescence intensity is measured using the Fluostar microarray reader and a graph is constructed from the data collected.

Purification of His tagged EGFP is done by thawing out the cell pellet, attained from expression, and preparing the AKTA prime for FPLC starts purification. The cell pellet is then suspended in 10ml of Extraction Buffer, sonicated, and then centrifuged at 8K rpms for 20 minutes. Once centrifuged, supernatant is collected in a 50ml Falcon tube, and cell pellet is stored at -20°C. A 20µl sample of the supernatant was taken and suspended in 20µl of sample buffer with 5% β-mercaptoethanol, while a small sample of the cell pellet was taken in suspended in 200µl of β-mercaptoethanol. Once the AKTA prime pumps have been cleaned, by running programs 8 and 9 (see His-Tag purification), with double d-H₂O, the column is then loaded with nickel, using program 10 (see His-Tag purification). Once loading is complete, the free nickel is washed from the column, using programs 8 or 10 (see His-Tag purification), by double d-H₂O. The protein sample is then injected into the system for binding, accomplished by running program 6 and placing pump A into Buffer A. Once all the protein is bound to the column the sample is eluted, placing pump B into Buffer B, by running program 7 (see His-Tag purification). Once eluted, samples are taken from selected tubes, containing the elution, to determine which tubes collected the protein. Aliquots (20µl) were taken from the tubes that protein was eluted into. The column was cleaned, using a 110mM EDTA and 1M NaCl solution,
by running program 10. The EDTA solution is removed from the column by running program 10 again, but placing pump A into deionized water. The pumps are finally cleaned, by running programs 8 and 9, while pumps A and B are in double deionized water. An SDS-PAGE was run for these aliquots to determine which tubes contain significant amounts of protein. The purified protein sample is dialyzed over three days, in 700ml of 10mM Tris and 1mM DTT buffer a day, to remove the imidazole. The tubes with smaller amounts of protein, indicated in the SDS-PAGE gel, are then combined into centriprep tubes and concentrated down. The protein samples, once concentrated, are then dialyzed in the same manner. Once dialysis is completed UV-Vis at 350nm is run for the dialysis solutions, taken after each day, and the protein sample.

Upon completion of purification followed by dialysis, UV-Vis was run, between the wavelengths 600 nm and 200 nm. The UV-Vis spectrum indicates that there is protein present in the elution. The concentration of protein recovered from purification was calculated at 90.3 µM in 8 ml of solution.

**His-Tag Purification**

AktaPrime His tag purification programs:

Program 8: Pump cleaning
0-20 mL at 0% B, 5 mL/min, 0 mL collection

Program 9: Pump cleaning
0-20 mL at 100% B, 5 mL/min, 0 mL collection

Program 10: Column loading
0-40 mL at 0% B, 5 mL/min, 0 mL collection

Program 6 Inject and Wash of His tagged Proteins
Parameters that remain unchanged are not repeated.

Set Method Base = min

Set Fraction Base = mL

Set Pressure Limit = 0.3 Mpa

Break Point Conditions
0.0 0%B
Flow Rate = 5 mL/min
0 mL fraction size
Buffer Valve Position = Pos1
Inject Valve Position = Load
Set Peak Collect = no
Autozero = no
Event Mark = no

10.0 % B  
Autozero = yes

11.0 % B
Inject Valve Position = Inject
Autozero = no

13.0 % B  
Inject Valve Position = Load

73.0 % B  
End Method

Program 7  Elution Gradient of His tagged Proteins
Parameters that remain unchanged are not repeated.

Set Method Base = min
Set Fraction Base = mL
Set Pressure Limit = 0.3 Mpa

<table>
<thead>
<tr>
<th>Break Point</th>
<th>Conditions</th>
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<tbody>
<tr>
<td>0.0</td>
<td>0% B</td>
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<tr>
<td></td>
<td>Flow Rate = 5 mL/min</td>
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<td></td>
<td>Fraction Size = 8 mL</td>
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<td>Inject Valve Position = Load</td>
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<tr>
<td></td>
<td>Set Peak Collect = no</td>
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<td>Autozero = no</td>
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<td></td>
<td>Event Mark = no</td>
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<tr>
<td>1.0</td>
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</tr>
<tr>
<td>3.0</td>
<td>10% B</td>
</tr>
<tr>
<td>40.0</td>
<td>100% B</td>
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</table>
Fraction Size = 0 mL

<p>| | |</p>
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<tbody>
<tr>
<td>42.0</td>
<td>100% B</td>
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<tr>
<td>45.0</td>
<td>0% B</td>
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</table>

**End Method**

**Purification of GFP 7E15 (D11) using gel filtration column**

The protein fractions were concentrated all to 4 mL with a concentration of 27mg/mL (818uM). The 4mL was injected into the filtration column and eluted using 10mM Tris buffer pH 7.4. Protein elution occurred and we lost ½ of the protein. We could finally obtained 30mg/mL of the protein (approximately 1.5mL). The protein was separated and one portion was diluted to 18mg/mL. The diluted protein was used for protein crystallography.

**Stock Buffer solution preparation for protein Crystallography:**

50 mL of each buffer was prepared in falcon tubes and were filtered using 0.45uM filters.

- 100 mM Hepes Buffer (pH 8.1-8.5)
- 50% PEG 4000 buffer
- 10mM 2-Mercapto Ethanol
- 500 mM MgCl$_2$

**Growing crystals for GFP 7E15**

Starting buffer conditions used for growth

50 mL of each buffer was prepared in falcon tubes and were filtered using 0.45uM filters.

- 100 mM Hepes Buffer (pH 8.1-8.5)
- 50% PEG 4000 buffer
- 10mM 2-Mercapto Ethanol
- 500 mM MgCl$_2$
APPENDIX B

Figures

1. Active/passive reporters

2. Gene markers

3. Flow cytometry

4. Protein folding and trafficking

5. Cell sorting

6. Gene Transfer and expression

General applications of fluorescent proteins.

Sequencing analysis of mCherry and GFP.
Electrostatic distribution on the surface of GFP.
Electrostatic distribution on the surface of mCherry.

IPTG Structure and molecular formula

IPTG is used as a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac-operon [6]. Unlike allolactose, the sulfur (S) atom creates a chemical bond which is non-hydrolysable by the cell, preventing the cell from “eating up” the inductant. Many regulatory elements of the lac operon are used in inducible recombinant protein systems.
IPTG action and GFP DNA plasmid.

Regulation of the *lac* Operon in *E. coli*
Although lactose is the normal substrate of the enzyme beta-galactosidase, the inducer molecular that binds to the lac repressor and inactivates its DNA binding protein is actually allolactose, a lactose related compound that is produced by basal levels of beta-galactosidas. Allactose levels increase when lactose levels increase, leading to repressor inactivation and transcription of the lactose operon. It is possible to artificially induce the lac operon using a nonmetabolizable allolactose analogue, isopropylthiogalactoside (IPTG), which binds to the lac repressor protein.
**Description**

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>BL21(DE3)</th>
<th>BL21(DE3)pLysS</th>
</tr>
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<tbody>
<tr>
<td>BL21</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>DE3</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>pLysS</td>
<td></td>
<td>X</td>
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<tr>
<td>Chloramphenicol resistance</td>
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</table>

Expression steps for the protein mCherry

- **Transformation**
  - BL21(DE3)

- **Inoculation**
  - Shake overnight at 37°C

- **Expression**
  - Shake at 37°C to desired $O.D_{600 \text{nm}}$

- **Take sample for analysis**
  - $O.D$ (600nm)
  - SDS-PAGE
  - 10 μL sample buffer +
  - 5% β-mercapto ethanol
  - Fluorescence measurement
  - 10mM TRIS (200 ul) pH 7.4

- **Centrifuge**
  - Collect pellet and store at low temperature

- **When $O.D_{600 \text{nm}} \approx 2.000$**
  - Collect cell pellet

- **Induce with 0.2mM IPTG when $O.D_{600 \text{nm}}$ is $\approx 0.600$**

*AMP = Ampicillin (inhibits the enzyme transpeptidase used for cell wall synthesis)
Expression steps for the Green Fluorescent Protein.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Probable Cause</th>
<th>Possible Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No or low expression</td>
<td>Insert ligated into wrong reading frame</td>
<td>Check sequence carefully and determine which vector, pRSET A, B, or C is appropriate with the restriction site selected</td>
</tr>
<tr>
<td>Kinetics of induction different than expected</td>
<td>Try a longer time course for induction than the 4-5 hours recommended</td>
<td></td>
</tr>
<tr>
<td>Not induced at OD\text{\textsubscript{600 nm}} 0.4-0.6</td>
<td>Induce expression at OD\text{\textsubscript{600 nm}} 0.4-0.6</td>
<td></td>
</tr>
<tr>
<td>IPTG solution is too old</td>
<td>Prepare a fresh solution of IPTG or use up to 10 mM IPTG</td>
<td></td>
</tr>
<tr>
<td>Protein is difficult to detect on a Coomassie-stained gel</td>
<td>Perform a western blot using the Anti-Xpress™ antibody for detection</td>
<td></td>
</tr>
</tbody>
</table>

Troubleshooting expression experiments.
OD(600) for comparing the cell growth of BL21(DE3) expressing different mCherry variants of mCherry. Inoculated growth was transferred to 1L LB/AMP media. 1mL aliquots were collected at different hours to measure OD600. Induced with 0.2 mM IPTG at OD ~0.6.
OD(600nm) and fluorescent intensity for the expression of the direct mutations MC-1 and MC-2 calcium sensors.
Expression of seven mutants of mCherry as a new group of calcium binding sensors. Graphs show the optical density (OD) absorbance at 600nm diluting (5X) and non-diluting the growth media during expression. Each liter was induced with 0.2mM IPTG after 4hrs of growth transfer.
Primer sequence for the development of two calcium binding pockets in mCherry using PYMOL. The titration of K198D by 1D-NMR was obtained from Jiang Yusheng.

Fluorescent emission spectra for mCherry based sensors K198D-MC1 (A and B) and A145E MC2 in the presence of different concentrations of calcium excited at 540nm. Fluorescence change for K198 is fitted using 1:1 equation with Kd of 160µM (Jiang Yusheng).
The analysis of the two fluorescent calcium binding sensors that exhibit fluorescent change upon binding calcium. This protein is not sensitive to pH change from 7.11 to 8.44. Its Kd calcium binding is approximately 160µM and fluorescent intensity of the protein increases upon the binding of 1mM calcium.
Absorbance spectrum from the combination of three elutes (Total= 18mL) of the protein GFP7E15 (G1). 200μL of protein was diluted into 800 μL of 10mM Tris buffer (pH 7.4) for absorbance analysis (5x fold).

Ca^{2+} response of mCherry (A) and its variants including M-I-115 (B), M-I-153 (C), M-I-169 (D), and M-I-187 (E) in UV/Visible absorbance spectra
Ca$^{2+}$ response of mCherry (A) and its variants including M-I-115 (B), M-I-153 (C), M-I-169 (D), and M-I-187 (E) in fluorescence spectra.
Appendix C

Expression and purification of Calsequestrin
Comparison of metal ion binding of mCherry (A) and its variants including M-I-115 (B), M-I-153 (C), M-I-169 (D), and M-I-187 (E) with 50 mM (blue ) and 100 mM ( pink) metal ions.

Calsequestrin (CSQ):
- Major calcium storage protein expressed in the SR
- Targeted to the junctional SR in the vicinity of the RyR
- Prevent $\text{Ca}^{2+}$ precipitation
- Facilitate efficient storage mostly in cardiac muscle
- Binding capacity of 40-50 $\text{Ca}^{2+}$ ions per protein.
- MW: Skeletal CSQ= 63KDa, Cardiac CSQ= 55KDa
- Regulation of cardiac excitation-contraction coupling.
- Linked to arrhythmias and sudden death induced by exercise and emotional stress

MW: $\sim$40 kDa;
Amino acid: $\sim$400
Ca-binding site: $\sim$50;
Ca-binding affinity: 1~100 $\mu$M;
Cation binding affinity: $\text{La}^{3+}$$\rightarrow$$\text{Zn}^{2+}$$\rightarrow$$\text{Cd}^{2+}$$\rightarrow$$\text{Mn}^{2+}$$\rightarrow$$\text{Ca}^{2+}$$\rightarrow$$\text{Mg}^{2+}$$\rightarrow$$\text{Sr}^{2+}$$\rightarrow$$K^+$.

Expression of CSQ using the cell line BL21(DE3) inducing with different concentrations of IPTG (0.2, 0.5, and 1mM respectively).

Expression of CSQ using the cell line Rosetta Plyss inducing with different concentrations of IPTG (0.2, 0.5, and 1mM respectively).

Expression and purification of Calsquestrin have been started and it seems that the best conditions for expression is with 0.5 mM-1mM IPTG addition with BL21-DE3 as the cell strain.
Expression and purification of CSQ using the cell line BL21(DE3).

401 Amino Acids: F=21, Y=13, W=5, MW=46KDa

Expression and purification of CSQ using the cell line BL21(DE3).