GENETICALLY ENGINEERED AEQUORIN FOR THE DEVELOPMENT OF NOVEL BIOANALYTICAL SYSTEMS

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By
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The ability to rationally or randomly modify proteins has expanded their employment in various bioanalytical applications. The bioluminescent protein, aequorin, has been employed as a reporter for decades due to its simplistic, non-hazardous nature and its high sensitivity of detection. More recently aequorin has been subject to spectral tuning. Techniques such as random and site-directed mutagenesis, the incorporation of coelenterazine analogues and the incorporation of non-natural amino acids have expanded the palette of aequorin by altering their emission wavelengths and/or half-lifes. Due to the increased diversity of aequorin, it can be used in multianalyte detection.

Although aequorin has been studied extensively and has been used as a reporter in a wide array of applications, it has never been employed as a reporter in systems that involve the splitting of aequorin. Herein we describe the splitting of aequorin in such a way where it becomes the reporter protein in the development of protein-based molecular switches. We have created two distinct protein switches by genetically inserting the glucose-binding protein and the sulfate-binding protein into the aequorin sequence, splitting it in such a manner that it allows for the selective detection of glucose and sulfate, respectively. In a separate investigation, we developed a bioluminescence inhibition binding assay for the detection of hydroxylated polychlorinated biphenyls. These systems have shown that they can be employed in the detection of the respective analyte in biological as well as in environmental samples, which demonstrated a sensitive, fast alternative approach to current methods for on-site screening.

Furthermore, we propose the rational design, preparation and use of truncated aequorin fragments in bioanalytical platforms such as multi-analyte detection, protein complementation assays and protein tagging assays based on our discovery that truncated aequorin retains partial bioluminescence emission. One such truncated aequorin demonstrated a large red shift in the emission maximum. It is envisioned that this new red-shifted truncated aequorin will find applications in multi-analyte detection. We anticipate that this work will lead to the discovery of additional functional truncated
aequorin fragments that can be employed in novel protein-protein interactions or protein folding systems.

KEYWORDS: Photoprotein, Molecular Switch, Inhibition Assays, Binding Proteins, Truncated Proteins

Krystal Teasley Hamorsky

April, 23 2011
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DISSERTATION

Krystal Teasley Hamorsky

The Graduate School
University of Kentucky
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For my nieces, Kallie, Jersey and Amaya
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the data. Dr. Patrizia Pazini deserves thanks for her analytical insight in chapters three and four and I appreciate her help in revising all chapters of my dissertation.

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CHAPTER ONE

INTRODUCTION

Biotechnology is a rapidly growing field with seemingly endless applications for environmental, biological and medical sciences. Genetic engineering, a focal point of modern biotechnology, is a sophisticated tool used for the creation of new biomolecules with unique and distinct properties. Genetic engineering has been instrumental in the creation of new molecular entities with unique characteristics, including genetically engineered medicines, knockout genes, analyte tracking and many more. The continuous evolution imposes a need for the design and development of novel biomedical and environmental detection systems. Diagnosis and treatment begin with reliable, efficient, selective and sensitive identification of environmental or biological markers. Herein, the focus is on the employment of genetic engineering for the development of modified aequorin, a photoprotein, for detection and tracking of virtually any molecule of interest. The ability to improve the performance of aequorin-based detection systems and expanding the nature of aequorin as a reporter will be discussed. Currently, employing aequorin as a reporter is limited to the use of the intact full-length apoprotein, therefore we intend to investigate split and truncated aequorin systems for molecule detection.

BIOLUMINESCENCE

The term bioluminescence is a word that originates from the Greek word, *bios*, for “living” and the Latin word, *lumos*, for “light” therefore it is defined as light emission from a living organism. It is a naturally occurring form of chemiluminescence where energy (40-60 kcal) is released from a chemical reaction in the form of visible light. Bioluminescence is known for its abundance and ubiquity given that it is found in 700 genera, 80% of which are marine organisms.\(^1\) There are many different types of organisms capable of emitting light ranging from bacteria and fungi, to mollusks, crustaceans, insects, fish and plants.\(^2,3\) This curious phenomenon was first described by Aristotle (384-322 BC) as the light emitted from decaying wood.\(^2\) Since then, scientists have studied bioluminescence in depth with the aim of understanding how it is produced. It is recognized that the functions of bioluminescence include communication, mating
and defense, and the emission of light is closely controlled by chemical and neurological mechanisms although some chemical mechanisms are still unknown.

There are two general types of bioluminescence. The first type involves a biochemical reaction where the total amount of the emitted light is directly proportional to the amount of an organic compound, known as luciferin, present in the organism. The light emitting reaction is catalyzed by luciferase, an enzyme responsible for the oxidation of luciferin resulting in the production of light. The second type of bioluminescence used by the photoproteins involves a different reaction mechanism where the total amount of light emitted was proportional to the amount of the photoprotein present. Herein the protein of interest, aequorin, is a photoprotein, therefore photoproteins will be discussed in more detail.

PHOTOPROTEINS

In 1961, Osamu Shimomura and his colleagues isolated and identified a new type of protein that was luminescent. Before this, all known bioluminescence reactions involved some type of a luciferin-luciferase reaction. The protein Shimomura and colleagues discovered was capable of emitting light in aqueous solutions when Ca$^{2+}$ was added, and unlike the known luciferin-luciferase systems, the total amount of light emitted was proportional to the amount of the protein present. The new protein, aequorin, was named after the genus of the jellyfish, Aequorea, from which it was isolated. Aequorin was initially thought to be an exceptional and perhaps unique protein but shortly after its discovery, Shimomura and Johnson unearthed another unusual bioluminescent protein in the parchment tubeworm Chaetopterus. The total light emitted was again proportional to the amount of the protein present, but unlike aequorin, this newly found protein was activated by peroxide rather than calcium. Since these two proteins did not follow any light producing mechanism known at the time, a new term, “photoprotein”, was introduced to denote the bioluminescent proteins capable of emitting light in proportion to the amount of the protein present. This proportionality makes a clear distinction between a photoprotein and a luciferin/luciferase system where the amount of light emitted is proportional to the amount of luciferin present. There are at least three different types of photoproteins based on how they are induced to produce
light. There are those such as aequorin and obelin which are calcium activated, photoproteins that are peroxide activated such as that from *Chaetopterus* and an ATP activated photoprotein from the *Luminodesmus* millipede.

Since the isolation of aequorin in 1961, several other photoproteins have been isolated, among these we can list obelin from *Obelia geniculata* [Knotted thread hydroid (Hydrozoa)],\(^7\) clytin (a.k.a. phialdin) from *Phialidium gregarium* [Hydrozoa],\(^8\) thalassicolin from *Thalassicola* sp. [Radiolarian],\(^9\) pholasin from *Pholas dactylus* [Common piddock, (Mollusk)],\(^10\) polynoidin from *Harmothoe lunulata* [Polynoid worm],\(^11\) mnemiopsin from *Mnemiopsis* sp. [Cnidarian],\(^12\) berovin from *Beroe ovata* [Ctenephore],\(^12\) symplectin from *Symplectoteuthis oualaniensis* [Purpleback Flying Squid (Cephalopod)],\(^13\) etc. There are also other unnamed photoproteins isolated from organisms such as *Chaetopterus variopedatus* [Parchment worm],\(^5\) *Ophiopsila californica* [Brittle star]\(^14\) and the only known photoprotein of terrestrial origin from the millipede *Luminodesmus sequoia*.\(^15\)

**AEQUORIN**

The calcium-regulated photoprotein, aequorin, is one of the most extensively studied photoproteins identified so far. Aequorin was isolated from *Aequorea Victoria*, (Figure 1.1) a hydrozoa in the phylum cnidaria. As far as is known, all cnidaria species that luminous use the imidazopyrazinone, coelenterazine, as their light-emitter. In fact, coelenterazine is the light emitter in at least nine phyla and is considered the most extensively used luciferin in the sea.\(^4\) Cnidarians are unable to produce their own coelenterazine therefore coelenterazine is a dietary requirement for bioluminescence to occur.\(^16\) Research suggests that coelenterazine is acquired from the cyclization of the tripeptide, Phe-Tyr-Tyr.\(^17\) Aequorin is composed of two distinct units, the apoprotein (apoaequorin) with an approximate molecular weight of 22 kDa, and a prosthetic group, coelenterazine (molecular weight of 423 g/mol)\(^18\) (Figure 1.2). The apoprotein assembles spontaneously with coelenterazine in the presence of molecular oxygen to form the functional photoprotein. Binding of Ca\(^{2+}\) to aequorin causes the photoprotein to undergo a conformational change resulting in the oxidation of coelenterazine through an excited state to produce coelenteramide with a concomitant release of CO\(_2\) and emission.
Figure 1.1. *Aequorea victoria*. Hydromedusae from which aequorin and the green fluorescent protein were isolated. Photo courtesy of http://www.biosci.ohiou.edu/faculty/currie/ocean/brandnewimages/.
Figure 1.2. Three-dimensional crystal structure of aequorin. Protein data bank structure 1EJ3. Visualized using Discovery Studio Visualizer 2.5.5.
of light. As the excited form of coelenterazine relaxes to the ground state there is an emission of blue light at a $\lambda_{\text{max}}$ of emission of $\sim 470$ nm (Figure 1.3).

To further understand the bioluminescence emission of aequorin the highly ordered structure must be dissected. Apoaequorin is composed of 189 amino acids\textsuperscript{19} which creates four EF-hand structural domains, I, II, III and IV (from the N to C terminus). EF hands are helix-turn-helix structural domains found in some calcium binding proteins. They are typically two perpendicular alpha helices connected by a small loop.\textsuperscript{20} The 3-D crystal structure\textsuperscript{18} demonstrates that in aequorin the EF-hands are oriented in such a way that EF-hand I and II are mouth to mouth with EF-hands III and IV creating a hydrophobic core (Figure 1.2). The hydrophobic core is approximately 600 Å and coelenterazine is housed within the hydrophobic core, non-covalently via Van der Waals, hydrophobic and through hydrogen bonds, with molecular oxygen. Coelenterazine is stabilized in the hydrophobic pocket by 21 interior hydrophobic resides including three vital triads of amino acids; each triad contains a histidine, a tryptophan and a tyrosine. Triad I is comprised of H16, W86 and Y82, triad II is comprised of H58, W108 and Y132 and triad III is comprised of H169, W173 and Y184. The side chains in triad I form hydrogen bonds to the hydroxyl group of the phenol attached to C6 of coelenterazine and the side chains of triad II forms hydrogen bonds to N1 of coelenterazine. The molecular oxygen is covalently bound to C2 of coelenterazine and Y184 of triad III forms hydrogen bonds with the oxygen, in addition W173 and H169 stabilize the carbonyl group on C3 of coelenterazine (Figure 1.4). The crystal structure also shows that there is a C-terminal helix after EF-hand IV that forms numerous hydrogen bonds holding the two halves of aequorin together.\textsuperscript{18} In fact, the C-terminal proline was found to be essential for the long term stability of coelenterazine.\textsuperscript{21}

Another structural point of interest is the calcium binding to the EF-hands of the aequorin molecule. All of the EF-hands of aequorin except EF-hand II are known to bind calcium. The loop between the two helixes of an EF-hand in calcium-binding proteins encompasses twelve neighboring amino acids residues with the amino acids at positions
Figure 1.3. Light emitting reaction mechanism of aequorin.
Figure 1.4. Three triads showing the main residues which stabilize coelenterazine. Histidines are in blue; Tyrosines are in red; tryptophans are in purple. Hydrogen bonds are shown by the green dotted lines.
1, 3, 5, 7, 9 and 12 coordinating to calcium\textsuperscript{22,23}; these side chains contain oxygen for calcium coordination. When comparing the three calcium binding sites on aequorin with other calcium binding proteins, it was found that the glycine at position six is highly conserved.\textsuperscript{24} Calcium binding is essential for bioluminescence emission therefore these regions should be conserved, however research has found that calcium binding to two of the three calcium binding EF-hands triggers bioluminescence.\textsuperscript{25} More recently, it was also reported that, EF-hand I has a lower affinity for calcium than EF-hands III and IV.\textsuperscript{26} Furthermore, it is important to note that other cations are capable of binding to EF-hands, in fact it has been demonstrated that the bioluminescence of aequorin can be triggered by Sr\textsuperscript{2+}, Cd\textsuperscript{2+}, Y\textsuperscript{3+} and La\textsuperscript{3+}.\textsuperscript{27} The complexity of the bioluminescence emission has been well studied and it has been thought that the structure is highly ordered therefore the use of aequorin as a reporter as been limited to employing full length apoaequorin. Herein, we discuss the existing employment of aequorin as a reporter as well as the future of exploiting aequorin as a reporter in split systems, truncated systems and inhibition assay systems.

**ADVANTAGES OF AEQUORIN AS A REPORTER**

The bioluminescence characteristics of aequorin allow it to be detected in the attomole range,\textsuperscript{28} making it a highly desirable reporter/label for many biological and analytical applications.\textsuperscript{29} Aequorin has found a niche as a reporter in certain applications where only radiolabels were previously used because it provides excellent sensitivity of detection while avoiding the risks associated with exposure, handling and disposal of radioactivity.\textsuperscript{30} Aequorin is quite stable, and under appropriate storage conditions it can retain its activity for years.\textsuperscript{31} Moreover, photoproteins offer significant advantages when employed as labels to detect biologically relevant molecules in biological fluids. Given that bioluminescence is quite rare in nature, and a light source for excitation is not required, any interference associated with the well-known background fluorescence of biological fluids is virtually eliminated.\textsuperscript{32} Also, unlike some chemiluminescent labels that require alkaline conditions of pH9 or greater for maximum activity, the photoproteins are most active at physiological pH.\textsuperscript{28} Furthermore, the possibility of using aequorin in an homogeneous format makes miniaturization possible for use in field-
TRADITIONAL APPLICATIONS OF AEQUORIN AS A REPORTER

These attractive properties have led to the employment of aequorin in numerous applications. The cloning of aequorin in 1985 by Tsuji et al. and Prasher et al.\textsuperscript{41,42} paved the way for its use as an intercellular Ca\textsuperscript{2+} indicator\textsuperscript{9,43,44}, since aequorin emits light in a stoichiometrically calcium dependent manner. Additionally, aequorin has been employed as a highly sensitive label in assays, mainly immunoassays and DNA hybridization assays.\textsuperscript{29,45}

A. Immunoassays

Immunoassays have been employed in the detection of a plethora of molecules, including diagnostic biomarkers. The major advantages of immunoassays over other detection methods are their specificity and sensitivity, which is conferred by the nature of the biological reagents employed, namely antigens, antibodies and signal generating molecules (labels). Immunoassays utilize the binding between an antigen and its antibody to quantify the target analyte in a given sample. The specific interaction between an antibody and its antigen provide immunoassays with their high degree of selectivity. In order to detect these interactions, it is important to employ signal generating compounds, such as aequorin that can be linked to the either the antigen or to the antibody. The most common linking methods used for bioluminescent molecules are chemical conjugation and gene fusion. Chemical conjugation can be performed in a number of different ways. If the antigen is a relatively small molecule it may be possible to functionalize it with a reactive linker that will attach to specific amino acids, typically the primary amines of lysines or the sulphydryl groups of cysteines, in the bioluminescent protein.\textsuperscript{46} In some cases, it may be desirable to attach the antigen to a single specific location on the bioluminescent protein in order to control the number of antigens attached and to prevent the loss of bioluminescent activity that might result should the antigen react with an amino acid essential for activity. In order to accomplish this type of site-specific labeling, it may be necessary to perform genetic modification of the bioluminescent protein so that it contains only one reactive site, for example, a unique
cysteine at a specific location. This methodology was employed by Lewis et al. for the
development of an aequorin-based assay for thyroxine. A bifunctional cross-linker that
reacts with different side chains, for example, cysteines on the antibody and lysines on
the bioluminescent protein, can be used for linking an antibody with a bioluminescent
protein.

An alternative to chemical conjugation for linking the bioluminescent protein to
the antigen or antibody is the creation of a fusion protein. Molecular biology techniques
can be used to fuse the gene encoding for a bioluminescent protein to the gene sequence
encoding for the antigen or an epitope of the antigen. The fused gene can then be
expressed, and the fusion protein purified. Gene fusion is superior to chemical
conjugation because the resulting fusion protein is more homogeneous in nature
compared to the products of chemical linking and because it is produced by recombinant
means, it should have identical performance after each production.

Immunoassays can be separated into two different categories, namely
heterogeneous or homogeneous. In the homogeneous format, the antigen or labeled
antigen to antibody binding occurs in solution where no separation of the two is
necessary. Thus, homogeneous assays can be referred to as one step or one phase assays.
In the heterogeneous format, the antibody is immobilized onto a surface, such as on the
walls of microtiter plate wells, on beads or membranes, where the reaction takes place.
In this assay format, the immobilized antibody, the antigen in solution and the sample are
mixed. Because the antibody is in the solid phase a separation step is required to
eliminate excess of reagent and sample prior to reading of the generated signal.
Homogeneous assays require no separation step, thus they are more rapid and convenient
than heterogeneous assays, whereas the latter are more sensitive. Heterogeneous and
homogenous assays can be further classified as being either competitive or
noncompetitive (Figure 1.5). In competitive immunoassays, the unlabeled antigen and
the labeled (i.e., aequorin) antigen compete for a limited number of antibody binding
sites. In this format, the signal of the label is inversely proportional to the amount of
analyte antigen present in the sample. Alternatively, in noncompetitive immunoassays,
Figure 1.5. Top: Heterogeneous competitive immunoassay. (a) A specific antibody (light blue) is immobilized onto a surface. (b) The unlabeled antigen (green sphere) and the labeled antigen (green sphere with red label) are added followed by a washing step. (c) After the washing step, the triggering buffer is added, and bioluminescence is detected. Bottom: Heterogeneous noncompetitive immunoassay. (a) A specific antibody (light blue) is immobilized onto a surface. (b) The antigen (green sphere) is bound to the antibody. (c) The bioluminescent labeled antibody (dark blue antibody with red label) is added, and the excess is washed away. (d) Following the wash step the triggering buffer is added, and bioluminescence is detected.
otherwise known as “sandwich” immunoassays, the analyte is bound to its antibody, and then a second labeled antibody is bound to the antigen-antibody complex. The resulting intensity of the label is directly proportional to the amount of analyte antigen present in the sample.

The majority of immunoassays that use aequorin as a reporter are of heterogeneous format, due to their higher sensitivity. A number of aequorin-based immunoassays have been developed for the detection of biologically relevant molecules such as, thyroxine, leu-enkephalin, cortisol, digoxin, biotin, angiotensin II, methamphetamine, prostacyclin and serotonin. Additionally, there are several commercially available bioluminescent immunoassay kits that are employed for rapid and sensitive clinical diagnostic tests. For example, the ChemFLASH™ Aqualite® Streptavidin Conjugate Pack is available. This pack is designed for the detection and quantification of any biotin-labeled target utilizing the aequorin in a “sandwich” type immunoassay (Chemicon (Millipore)).

B. DNA Hybridization Assays

Nucleic acid hybridization assays are powerful bioanalytical tools for the identification of specific sequences of DNA or RNA. The highly specific and strong interaction between two complementary nucleic acid strands forms the basis for DNA hybridization assays. In these types of assays, scientists use a labeled gene probe that is complementary to the target gene of interest. Historically, the field of hybridization assays has been dominated by the use of radioactive labels such as phosphorus, sulfur and hydrogen. This is due to the fact that the amount of DNA present in a given cell is very minute, 0.1 pg to 6.0 pg, depending on the organism. In order to be able to detect such small amounts of DNA the assays have to be very sensitive. In the past twenty years, however, there has been a trend to replace radioactive labels with chemiluminescent and bioluminescent labels due to the excellent sensitivity and their lack of toxicity.
Labeled nucleic acid probes are utilized in a variety of assay formats including homogeneous assays and heterogeneous assays (similar to immunoassays), Southern blots, Northern blots and dot blots. In Southern blot analysis, the DNA of interest is treated with restriction enzymes which cut the DNA at specific positions. The restricted pieces of DNA are then run on an electrophoresis gel to separate the DNA fragments according to their size. The DNA is then denatured, either chemically or by heat, followed by the transfer to an appropriate membrane. The labeled probe which was designed to bind to a certain DNA fragment is then incubated with the membrane. After the hybridization is complete, the membrane is washed and visualized. In Southern blot analysis the material detected is the isolated DNA, whereas in Northern blots RNA is detected. Northern blots employ the same steps as Southern blots, except that the isolated genetic material is RNA. In dot blot analysis, gene probes are employed in the same manner as in Southern and Northern blots, however, the DNA or RNA is not separated electrophoretically, instead it is spotted onto the membrane directly followed by visualization.

There are three main configurations for the bioluminescence detection of nucleic acid sequences by using hybridization assays (Figure 1.6). In the first configuration, “the immobilized target assay”, the target DNA or RNA is immobilized to a solid surface, such as beads or microtiter plate walls. After the immobilized DNA is denatured by chemical methods, such as treating with NaOH, the target DNA is hybridized with a specific probe that is linked to a bioluminescent label. In the second method, “the immobilized probe assay”, the target DNA is denatured and then hybridized with a probe that was immobilized on the solid surface. The hybridized target is then labeled with the bioluminescent label (i.e. aequorin). The target DNA may be labeled with the polymerase chain reaction (PCR) by using a primer biotinylated at the 5’ end or by incorporating biotin labeled deoxyribonucleotides (dNTPs). The bioluminescent label, conjugated to streptavidin, is then added to the mixture. Due to the strong interaction between the biotin and streptavidin molecules, the target DNA is labeled with the bioluminescent reporter. The third configuration is the classical “Sandwich-type assay”. The target DNA is denatured and then hybridized with two different probes.
Figure 1.6. Schematic representation of the three different hybridization assay configurations as discussed above in the text. (a) “Immobilized target” assay - where the target DNA is immobilized on a surface and detected by a labeled probe. (b) “Immobilized probe” assay - where the probe is immobilized on a surface and incubated with the labeled, target DNA. (c) “Sandwich-type” assay - in which a probe is immobilized on a surface then the target DNA is hybridized and finally the target DNA is detected with a second labeled probe.
probes is immobilized on a solid surface and the other is linked to the bioluminescent label. This final method is usually more specific since the target DNA has to hybridize with two different probes.\textsuperscript{64}

Aequorin-based DNA hybridization assays have been established for the detection of pathogenic organisms, such as \textit{Salmonella},\textsuperscript{65} pseudorabies virus,\textsuperscript{66} hepatitis B,\textsuperscript{67} \textit{Plasmodium falciparum},\textsuperscript{68} \textit{Escherichia coli}\textsuperscript{67} and \textit{Shigella sonnei}.\textsuperscript{37} DNA hybridization assays have benefited from the use of aequorin as a reporter molecule as it yields more sensitive assays than those based on other types of luminescent labels.\textsuperscript{69} Furthermore, since their detection is rapid, hazard-free and can be easily adapted to formats that are automated, aequorin-based DNA probes are gaining popularity in the detection of nucleic acids.

**DESIGNER PHOTOPROTEINS**

One factor that limits the more widespread use of photoproteins is their bioluminescence emission characteristics. Compared to the green fluorescent protein (GFP) and its variants, whose emission wavelengths range from blue all the way to red, the emission range of the photoproteins is much more limited. The bioluminescence emission maxima of the native photoproteins range between the 440 nm peak of thalassicolin to the 510 nm peak of polynoidin.\textsuperscript{31} Even though a 70 nm difference in emission is enough to use in multi-analyte detection, many applications would greatly benefit if there was a wider range of emission wavelengths to select from.

The green fluorescent protein (GFP)\textsuperscript{70} from \textit{Aequorea} and red fluorescent protein from \textit{Discosoma} sp. (dsRed) have been extensively studied, manipulated and mutated to create a multitude of variants capable of emitting fluorescent light in many different colors\textsuperscript{71-73} and demonstrating varied stabilities\textsuperscript{74,75} and maturation half-lives.\textsuperscript{76,77} Unfortunately, aequorin has not gone through similar manipulation to the extent that the fluorescent proteins have, and therefore, the varieties and its applications are more limited. To date, only a few reports have emerged that focus on designing photoproteins with altered bioluminescence emission. These include works that describe pairing different coelenterazine analogues with aequorin, ways of creating bioluminescence
resonance energy transfer (BRET) reactions using aequorin and employing random and rational mutagenesis as well as incorporating non-natural amino acids. The following sections review recent advances in the design of aequorin mutants with fine tuned bioluminescence characteristics, such as emission maxima, stability, activity, reduced cross-reactivity and decay kinetics. It is envisioned that increasing the spectral diversity of aequorin will allow for an increase in its employment as a reporter in multi-analyte detection and imaging.

A. Incorporation of Coelenterazine Analogues: Semi-Synthetic Aequorins

Perhaps the easiest approach for tuning the emission characteristics of the bioluminescence reaction of aequorin is to substitute the native coelenterazine with synthetic coelenterazine analogues (Table 1.1). Synthetic coelenterazines have been used to produce aequorins with different Ca\(^{2+}\) sensitivities, varying regeneration times, altered emission kinetics and different bioluminescence emission wavelengths. Shimomura et al. tested 32 different coelenterazine analogues paired with recombinant aequorin and determined the time it takes to generate 50% of the maximum aequorin activity. The results showed that the regeneration time for these semi-synthetic aequorins varied from 8 minutes for coelenterazine-\(e\) to 300 minutes for coelenterazine-\(n\). In a similar work, Shimomura et al. tested the sensitivity of aequorin charged with each of the 32 synthetic coelenterazines towards Ca\(^{2+}\) ions and found that there was a 19,000-fold range in the Ca\(^{2+}\) sensitivities of the semi-synthetic aequorins. When luminescence was triggered with a low concentration of Ca\(^{2+}\) (0.1-1 \(\mu\)M Ca\(^{2+}\)) in a low ionic strength buffer, the intensity of light was proportional to the square of the Ca\(^{2+}\) concentration. The intensities of the tested coelenterazine analogues ranged from 0.01 relative light units (RLU) for coelenterazine-\(n\) to 190 RLU for coelenterazine-\(hcp\). The same semi-synthetic aequorins were also measured for their decay kinetics. Their half-lives were found to vary between 0.15 s for coelenterazine-\(hcp\) to 8 s for coelenterazine-\(i\). In addition, these studies showed that the bioluminescence emission maxima ranged from 438 nm for the coelenterazine analogue (\(I\)) (Figure 1.7) to 476 nm for coelenterazine-\(i\).

B. Bioluminescence Resonance Energy Transfer
Table 1.1. Synthetic coelenterazine analogues. ntv stands for native coelenterazine.
Figure 1.7. Structure of coelenterazine analogue (I).
Scientists, inspired by nature, have tried to couple aequorin to natural or synthetic fluorophores in order to shift the emission wavelength towards the red end of the spectrum by employing bioluminescence resonance energy transfer (BRET). In the jellyfish, the light emitted by the bioluminescence reaction of aequorin is transferred to green fluorescent protein (GFP) and the emission wavelength is shifted from blue (469 nm) to green (508 nm). By joining the genes for aequorin and GFP Gorokhovatsky et al. were able to create a fusion protein of aequorin and GFP joined by a cleavable 19 amino acid long peptide spacer. Upon addition of Ca\(^{2+}\) to trigger the bioluminescence, they observed that the intensity of the bioluminescence emission decreased while a new peak corresponding to the fluorescence emission wavelength of GFP appeared. Also, the reversal of this observation was reported when the spacer between the aequorin and GFP was cleaved. This was evidence that BRET was responsible for the emission shift in this system since the spatial proximity of the donor molecule (aequorin) to the acceptor molecule (GFP) was confirmed to be essential for the energy transfer.

Another example of the successful use of BRET was demonstrated by Shimomura et al. using a synthetic fluorophore, carboxyfluorescein. Carboxyfluorescein succinimidyl ester (CFSE) was reacted with aequorin resulting in the conjugation of the carboxyfluorescein molecules to the aequorin through the protein’s lysine residues. The resulting aequorin-CFSE conjugate had an emission maximum of 528 nm. The emission intensity was twice of that the aequorin bioluminescence and had a comparable decay half-life of between 0.6-1.2 s demonstrating that much of the energy from the bioluminescence was being converted to fluorescence.

This conjugation of carboxyfluorescein to aequorin proved that through BRET the emission wavelength of aequorin bioluminescence could be shifted. However, the conjugation of the fluorophore to aequorin was not site-specific and since aequorin contains 14 solvent accessible lysine residues, this kind of approach results in more than one molecule of carboxyfluorescein conjugated to aequorin. This can produce batch-to-batch variations in the preparation of the conjugated aequorin giving results that are
variable or not reproducible. In order to overcome this drawback, Deo et al. used a previously prepared mutant of aequorin that does not contain any cysteine residues and has higher bioluminescent activity as the template for site-directed mutagenesis. Starting with this cysteine-free mutant, Deo et al. prepared four different unique aequorin mutants containing single cysteine residues. These mutants were A69C, G70C, G74C and G76C and the positions of these residues were selected due to their proximity to the location of the bound coelenterazine. These mutants were then individually conjugated to either N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD ester) or an iodoacetamide derivative of Lucifer Yellow. The results showed that when the amino acid positions 74 and 76 were labeled with either fluorophore, no BRET was observed. The reason for this, as explained by the authors, is that even though the fluorophore was close enough to the chromophore for a successful energy transfer, the lack of fluorescence was due to the improper orientation of the fluorophore for an efficient dipole-dipole interaction. On the other hand, when residues 69 and 70 were labeled, both conjugates showed an efficient BRET and an increase in the emission intensity corresponding to the emission maximum of the conjugated fluorophore ($\lambda_{\text{max}}=536$ nm for IANDB and $\lambda_{\text{max}}=531$ nm for Lucifer Yellow) was observed.

C. Random Mutagenesis

Another logical approach for producing more desirable variants of aequorin is random mutagenesis. In one study, Tsuzuki et al. used random mutagenesis and in vitro evolution based on DNA shuffling to isolate aequorin mutants that showed a change in thermostability. Specifically, the cDNA of apoaequorin was digested with DNase I, and the DNA fragments were subjected to DNA shuffling. The bacterial colonies were screened in microtiter plates and colonies that showed luminescence activity were selected. The DNA of these colonies was amplified and the amplified DNA was used for a second round of DNA shuffling. After three rounds of DNA shuffling the brightest light emitting colonies were incubated at different temperatures and their activities were measured. The researchers found that out of 82 mutants that retained activity, 9 of them showed different thermostabilities. Two mutants, Q168R and L170I, exhibited an increase in protein lifetime at 37 °C. Further analysis showed that these mutants
increased aequorin thermostability. Conversely, a mutant, F149S, was shown to decrease the thermostability of aequorin.

In another study, Tricoire et al. subjected aequorin to DNA shuffling and reported aequorin mutants with different decay kinetics and calcium sensitivities. Following a procedure similar to the one outlined above, Tricoire isolated nine different aequorin mutants that have half-lives ranging from 0.7 s for a Q168R mutant to 39.9 s for E35G. They also demonstrated that each mutation that affected the decay-rate was located either within or in close proximity of one of the three calcium binding domains of aequorin. From these mutational studies, they found that even though each individual EF-hand is sufficient to trigger luminescence they exhibit different affinities towards Ca\(^{2+}\). They also showed that the calcium EF1- and EF3-hands were more important for the kinetics of the bioluminescence decay than the EF4-hand and that the intensity of the luminescence was inversely proportional to the decay half-life of the same mutant.

D. Site-Directed Mutagenesis

One of the most powerful tools in protein engineering is site-directed mutagenesis where one or more nucleotides in the protein’s coding sequence is changed in order to alter the amino acid sequence in the protein. By using this powerful tool scientists can study the relationship between the structure and the function of a particular protein. The early site-directed mutagenesis studies on aequorin were to gain information about its structure-function relationships. In 1986, Tsuji et al. performed site-directed mutagenesis and replaced the three cysteine residues, three glycine residues and one histidine residue. The mutations of glycines within the EF-hand regions demonstrated that the G158R mutant did not result in a loss of bioluminescence emission, suggesting that the binding of calcium to EF-hand IV is not essential for emission or the change to arginine does not affect the binding of calcium. Furthermore, they reported that histidine 58 was involved within the active site. Other studies found that a C-terminal proline and the histidine residue at position 169 were both essential for its activity, and the tryptophan residue at position 86 was involved in the bioluminescence emission. The wild-type AEQ contains three cysteine residues at positions 145, 152 and 180, however it
was found that removing all of the cysteines yields a mutant with increased bioluminescence\textsuperscript{93}. In addition, without the cysteine residues there is no need for the addition of a reducing agent for incubation with coelenterazine therefore this cysteine-free mutant has been employed in many assays.

In 2000, when the crystal structure of aequorin was made available,\textsuperscript{18} many researchers started performing site-directed mutagenesis in order to introduce desirable characteristics into aequorin. Armed with the knowledge of which particular amino acids are involved within the active site, scientists started engineering new aequorin mutants to suit their needs. Then in 2005, Stepanyuk et al. reported that after the mutation of the Y82 in aequorin to phenylalanine, the H-bond that stabilizes the chromophore is removed.\textsuperscript{94} This resulted in a change of the spectral properties of aequorin to resemble that of the photoprotein obelin, in other words the emission peak was red-shifted. In that paper, a shift in the bioluminescence emission wavelength of aequorin from 469 nm to 501 nm was observed. This result was later confirmed by Dikici \textit{et al.}\textsuperscript{95} In that work, site directed mutagenesis studies were performed on aequorin generating several mutants with different emission wavelengths ranging from 466 nm to 494 nm when paired with native coelenterazine.

\textbf{E. Multianalyte Detection}

It is only logical to assume that these new mutants when coupled with different coelenterazine analogues should give even better separation in their bioluminescence emission spectra. This hypothesis was demonstrated by Dikici \textit{et al.} when mutants of aequorin paired with different coelenterazine analogues resulted in as much as 74 nm of separation between the emission maxima of two mutants, W86F mutant coupled with coelenterazine-\textit{hcp} (445 nm) and Y82F mutant coupled with coelenterazine-\textit{i} (519 nm) (Figure 1.8).\textsuperscript{95} Because of this separation these mutants could be employed in dual analyte detection based on wavelength resolution. In the same paper, the authors also described bioluminescence emission decay half-lives of the mutants ranging from 0.23 s to 50.1 seconds. This combination of mutants with 0.23 s and 50.1 s half-lives represents an excellent opportunity for time resolved dual analyte detection. These new
Figure 1.8. Bioluminescence emission spectra of (—) cysteine-free aequorin with native coelenterazine (----), the aequorin mutant W86F with coelenterazine hcp and (---) aequorin mutant Y82F with coelenterazine i. Reprinted with permission from reference 95.
photoproteins can be employed in dual analyte detection. Rowe et al. developed a dual analyte detection bioluminescent immunoassay employing genetically modified aequorins as labels. They demonstrated an assay that can simultaneously detect two cardiovascular markers, 6-keto-prostaglandin-F1-α and angiotensin-II, by employing luminescence time resolution. In this assay, a 6-keto-prostaglandin-F1-α-aequorin conjugate and an angiotensin II-aequorin fusion protein were combined with coelenterazine and coelenterazine $i$ respectively, to form semi-synthetic aequorin variants. Time resolution was employed to resolve the signal of the two variants by the native differences in their decay kinetics and half-lives. Figure 1.9 shows the time resolution where the 6-keto-prostaglandin-F1-α signal was calculated from 0 to 6 s and the angiotensin II signal was calculated from 6.01 to 25 s. This method opens up new opportunities for multiplexing assays using the time resolution of aequorin signals to detect multiple analytes. In addition, two different bioluminescent labels can be used to develop multi-analyte immunoassays. Aequorin and firefly luciferase were employed to simultaneously detect prostatic acid phosphatase and prostate specific antigen or prostate specific antigen and fetoprotein.

F. Non-natural Amino Acids

An exciting new method of engineering proteins incorporates non-natural amino acids, which are not present in the native organism, into the protein structure. Incorporation of non-natural amino acids is an alternative method to altering the function of a protein. There are two different approaches for the incorporation of non-natural amino acids into the proteins, site-specific and global incorporation. Global incorporation involves the normal translation process of the cell and leads to the global incorporation of the non-natural amino acids into the protein structure. Site-specific incorporation directs the non-natural amino acid to a desired location by utilizing an orthogonal nonsense codon/tRNA method developed by Peter Schultz. While global incorporation is more facile, site-specific incorporation of non-natural amino acids allows for the precise positioning of the desired non-natural amino acid. Recently, it was reported that non-natural amino acids, 4-bromo-F-phenylalanine, 4-iodo-F-phenylalanine,
Figure 1.9. Bioluminescence decay kinetics profiles of 6-keto PGF1R aequorin coelenterazine (native) (black line) and angiotensin II-aequorin-coelenterazine-i (gray line) conjugates showing the flash versus glow-type decay kinetics of the two conjugates. The graph demonstrates how all of the bioluminescent signal of the 6-keto-PGF1R-aequorin- coelenterazine (native) conjugate is expired by the end of the 0-6 s time channel, leaving only the angiotensin II-aequorin- coelenterazine-i signal in the 6.01-25 s time channel. Reprint with permission from reference 96.
4-amino-F-phenylalanine and 4-methoxy-F-phenylalanine, were inserted into the cysteine-free mutant of aequorin at position 82, and the bioluminescence characteristics were determined to be altered. Most notable it was found that the incorporation of all four non-natural amino acids causes a red-shift in emission with native coelenterazine. Moreover, 4-methoxy-F-phenylalanine-82-aequorin paired with coelenterazine i had the furthest red shift having a 517 nm emission peak.\textsuperscript{100} This work opens up a whole new avenue for aequorin with the possibility of generating a whole palette of mutants for bioanalytical applications.

**FUTURE APPLICATIONS OF AEQUORIN**

Until now the employment of aequorin and its variants as reporters has been limited to the full-length apoaequorin. The ability to split or truncate aequorin will lead to a plethora of novel aequorin-based applications. The applications considered here, are split aequorin as the signaling molecule in protein switch sensing systems, as split non-self-assembling fragments for the detection of protein-protein interactions (PPIs) and as split self-assembling fragments for protein tagging. Additionally, truncated auto-luminescence fragments will be studied with the goal of discovering bioluminescent truncated fragments with changes in the bioluminescence characteristics, i.e., red-shifted emission maxima (explained above in the designer photoproteins section).

**A. PROTEIN SWITCHES**

Genetic engineering tools have been instrumental in the creation of new molecular entities with unique characteristics. The ability to rationally or non-rationally insert the gene of one protein into another has allowed for the creation of a new class of molecules called protein switches. Protein switches are defined as the fusion of two individual proteins creating a unique function that is regulated by an external signal. Protein switches contain both the receptor and transducer in a single polypeptide chain\textsuperscript{101} creating a much simpler system. The system is homogeneous in nature with no immobilization or washing steps necessary. Protein switches must contain a protein that will bind to an analyte causing a conformation change and a protein capable of emitting a detectable signal. Protein switches are being studied for purposes of understanding natural switches, to help explain protein form and function, to develop tools for
elucidating cellular function and behavior and to create switches for sensing and biomedical applications.\textsuperscript{102}

Of interest here are optical-based protein switches capable of detecting and monitoring specific molecules. Employing optical proteins as the signaling molecule is superior to using other labels, specifically radioactive labels, because optical proteins are derived from nature, non-hazardous, sensitive and biocompatible. Protein switches based on split fluorescent proteins have been studied for over ten years.\textsuperscript{101,103-108} For example, Baird et al. genetically inserted the calcium binding protein calmodulin into enhanced yellow fluorescent protein for the detection of calcium. This system has the potential to monitor cytosolic calcium in mammalian single cells.\textsuperscript{103} These fluorescent based systems have several advantages, including sensitivity and biocompatibility, however they suffer from photobleaching and background scattering. To overcome these limitations bioluminescent proteins can be used to generate the optical output, yielding lower background, enhanced sensitivity and less expensive instrumentation. To that end, we intend to develop optical protein switches for analytical sensing employing aequorin as the signal generating molecule.

Being able to split aequorin provides a gateway to expand the applications of the photoproteins. Discovering that proteins can be inserted into a photoprotein could allow for the development of protein switches with enhanced characteristics compared to other types of sensing systems. Current use of photoproteins as labels requires chemical conjugation or end-to-end gene fusion. Chemical conjugation techniques are difficult, can cause a loss in protein function and can result in variable performance. End to end gene fusion has the advantage of creating one to one conjugates with enhanced sensitivity and performance. However, end to end fusions many not be the most optimal way of linking two individual proteins to maximize the coupling of their functions. Protein switches generate fusions that can have enhanced characteristics due to the fact that one gene is inserted into the other. Kin \textit{et al.} provide evidence that protein switches can improve kinetic stability over end to end fusion.\textsuperscript{109} In addition, protein switch design allows a more versatile combination of functional fusion proteins\textsuperscript{101} and conformational changes.
in split fusion systems can show more of a dramatic change in structure and function. Rational design of protein switches allows for what researchers believe may be the most optimal switch, however, random fusion combinations may provide another avenue to optimal and enhanced systems. Random switch design can be accomplished via circular permutation (domain insertion) to create the best switch. Domain insertion has been studied in the creation of various molecular switches. Researchers hypothesize that using circular permutation in the creation of random bioluminescent protein switches will expand their versatility and create superior systems.

1. HINGE-MOTION BINDING PROTEINS

The development of optical molecular switches for sensing devices requires both a recognition and a reporting element. As discussed above the reporting component can be of an optical nature, encompassing fluorescent and bioluminescent proteins, and the recognition component requires a binding event to occur that can be transduced to the signal generating a distinguishable change in signal of the reporter protein. An ideal recognition module is one that has exquisite selectivity towards its ligand therefore the sensing switch will be analytically selective. Hinge-motion binding proteins are a superb choice given that they exhibit excellent selectivity towards their ligand. Hinge-motion binding proteins are a collection of proteins that resemble the mechanism of a Venus fly trap; having two domains connecting by a hinge which are in the open conformation when no ligand is present but in the closed form in the presence of its ligand. Hinge-motion binding proteins encompass many different classes of proteins however herein the focus is on periplasmic binding proteins.

Generally found in the periplasm of gram negative bacteria, periplasmic binding proteins are responsible for the uptake and transport of nutrients, sugars, oxyanions, amino acids, oligopeptides and other nutrients, from the periplasm to the cytoplasm of the bacteria. They are categorized according to the type of ligand, ions, carbohydrates, etc, to which it binds, and even though some of the ligands are totally unrelated, the binding affinities between the ligand and its corresponding periplasmic protein are similar. Periplasmic binding proteins consist of a single polypeptide chain with a
range of molecular masses, 23-59 kDa. The amino acid sequences of these proteins have little similarity,\textsuperscript{114} although the 3-D structures of periplasmic binding proteins are similar. The proteins are composed of an N-terminal domain and a C-terminal domain, and these domains are connected by two or three peptide strands which forms a cleft where the ligand binds.\textsuperscript{115} When the protein is not bound to the ligand the protein is considered open but when the ligand is present, the two domains engulfs the ligand causing the protein to be in the closed conformation (Figure 1.10).

This conformational change, when a ligand binds to its periplasmic binding protein, has been employed to create selective sensing systems for many ligands including sulfate, glucose and many more.\textsuperscript{113} Methods of developing these periplasmic-binding protein sensing systems vary and are discussed in a recent review by Moschou et al.\textsuperscript{113} For example, one popular method to create such sensing systems is to label the protein with an environmentally sensitive fluorophore so that when the ligand binds to the protein, the environment around the binding site is altered, causing a change in the fluorescence intensity of the fluorophore. In order to maximize the change in fluorescence, the fluorophore has to be in close proximity of the conformational change. Therefore researchers have rationally labeled binding proteins near their binding sites, with fluorophores, so the amount of ligand bound can be quantified by the changes in the fluorescence signal. While these systems are great sensing systems, they suffer from photobleaching and are not as applicable \textit{in vivo} as bioluminescent proteins due to background scattering caused by the incident light. To that end, we hypothesize that joining a periplasmic binding protein and split aequorin in a switching fashion can result in a sensing system that has both superb selectivity and excellent sensitivity.

\section*{B. PROTEIN-PROTEIN INTERACTIONS (PPIs)}

The cell is a complex circuit and most cellular functions are controlled by protein-protein interactions (PPIs). As a result, the \textit{in vivo} identification, characterization and localization of PPIs are indispensable to understanding processes in living organisms. Being able to understand how proteins interact begins with detection of the interactions
Figure 1.10. A) The 3-D crystal structure of GBP without glucose bound (Protein Data Bank – 2FW0). The “open” conformation. B.) The 3-D crystal structure of GBP with glucose bound (Protein Data Bank – 2GBP). The “closed” conformation. Visualized using Discovery Studio Visualizer 2.5.5.
which allows for further investigation. Currently there are several methods for detecting the ultimate goal of detecting PPIs, and amenable to high throughput experiments. However there is still opportunity for improvement given that each of the methods suffer from some limitation, therefore no one method can detect every possible PPI, especially under its native conditions, i.e. living cells. To date the most promising methods are the protein complementation assays (PCAs), also known as biomolecular complementation (BiC) assays, because they are able to monitor PPIs \textit{in vivo}, which is PCAs are based on rationally designed split reporter fragments that are genetically fused to two binding partners, bait and prey. When the bait and prey interact, the two reporter fragments reassemble forming an active unit which allows for the detection of the interaction (Figure 1.11). PCAs require employing a split reporter that does not associate spontaneously, because this would cause false positives. Furthermore, appropriate controls should be designed to monitor self-assembly of the reporter to ensure only interacting partners are being detected. To avoid self-assembly it is suggested to express the fusion proteins at low levels.

The existing toolbox of PCA reporters includes only β-lactamase, β-galactosidase, dihydrofolate reductase, fluorescent proteins, ubiquitin, luminescent proteins and TEV protease, and the characteristics of these reporters are tabulated in Table 1.2. Fluorescent or luminescent proteins allow for straightforward \textit{in vivo} imaging consequently they are more commonly used. Fluorescent proteins are more appropriate for localization information where as luminescent proteins are better suited for dynamic information. Fluorescent based PCAs, also known as biomolecular fluorescence complementation (BiFC), show great promise because they are simple reporters, requiring no substrate and can detect weak interactions \textit{in vivo} however because they are irreversible and chromophore formation takes time, interactions cannot be tracked directly and the presence of antagonists cannot be detected. A wide array of BiFCs have been developed and are discussed in recent reviews.
Figure 1.11. Illustration of a protein complementation assay. Left: The bait and prey do not interact therefore the reporter fragments do not interact. Right: The bait and prey are interacting partners therefore the two reporter fragments interact causing a detectable signal.
Table 1.2. Characteristics of split reporters used in protein complementation assays. This table highlights the applications of each reporter as well as the organisms that the reports are capable of performing in. Remade from reference 118.

<table>
<thead>
<tr>
<th>Reporter protein</th>
<th>Application</th>
<th>Organism</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Localization of PPIs</td>
<td>Dynamics</td>
</tr>
<tr>
<td>β-lactamase</td>
<td>×</td>
<td>Limited</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>Dihydrofolate reductase (DHFR)</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>Fluorescent proteins (FPs)</td>
<td>✓</td>
<td>Limited</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>Luminescent proteins</td>
<td>Limited</td>
<td>✓</td>
</tr>
<tr>
<td>TEV protease</td>
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Herein, we spotlight luminescent based PCAs (BiLC) given that we visualize developing BiLCs employing aequorin. Most commonly, luciferases rather than photoproteins have been used as reporters for BiLC assays. Luminescent proteins require a substrate therefore they are not as straightforward as fluorescent proteins, however they are reversible. Reversibility allows for the detection of inhibitor disruption and studies of PPI dynamics. Currently, BiLCs do not provide subcellular localization information but they are more suitable, than their fluorescent counterparts, for the detection of PPIs in living animals due to the virtually non-existent background. Several BiLC assays have been developed utilizing firefly luciferase, renilla luciferase, click beetle luciferase and Gaussian luciferase. In a recent example, Misawa et al. generated cells line that express G-protein coupled receptors (GPCR) and β-arrestin linked to split click beetle luciferase fragments to develop a rapid and sensitive BiLC in cultured mammalian cells. BiFC and BiLC development is on the up rise and shows a tremendous amount of promise, however there is still room to improve the characteristics of PCAs such as improved sensitivity, more spectral variations and diminished self-assembly.

Recently, multicolor BiFC and BiLCs have been reported which allow for visualization of multiple interactions between different proteins in the same cell. Hu and Kerppola originally reported simultaneous visualization of multiple PPIs using split fluorescent protein fragments with different spectral characteristics. They found twelve biomolecular fluorescent complexes, using GFP and its variants that yielded seven different spectral classes which can be used for spectral resolution. These variants were characterized by utilizing known interactions of the leucine zipper family proteins and two interactions were detected in parallel. In another study, split click beetle luciferase fragments were generated via random mutagenesis and cross complementation with different PPIs generated different emission maxima. These fragments were used for dual imaging of kinase-induced interactions of Smad1-Smad4 and Smad2-Smad4 in *Xenopus laevis* embryos. This method allows for real time and reversible simultaneous imaging of two different PPIs. Furthermore, recently there has been interest in combining PCAs with
resonance energy transfer techniques (BRET or FRET) which has allowed for identifying and imaging ternary protein complexes, i.e., GPCR signaling complex. The majority of PCAs only allow for the detection of two PPIs simultaneously and detection of multiple PPIs is more desirable. In theory, the number of interactions to be detected concurrently is unlimited however in reality the number is limited by the lack of spectral diversity of the reporter proteins. To that end, scientist must strive to develop more PCA reporters with greater spectral variation.

C. PROTEIN TAGGING

Protein fragments that self-assemble have the opportunity to be employed as reporters in protein tagging, which can be used to monitor protein folding and stability. An ideal tag is one that is sensitive, genetically encoded, works in vivo and in vitro, is reagentless and minimizes perturbation of protein folding and solubility. Protein misfolding and aggregation is the basis of numerous human diseases including Alzheimer’s disease, Creutzfeld-Jakob disease, Huntington’s disease and many more. The discovery of drugs that inhibit aggregation are vital and methodologies that can identify such drugs are desirable. Protein tagging presents a promising technique to identify protein misfolding in vivo however it requires having a split reporter protein that will spontaneously self-assemble. In a protein tagging system, the protein of interest is genetically fused to one fragment of the split reporter and the other half of the split reporter is expressed separately. When combined, the split reporter fragments self-assemble to create a signal only if the tag is accessible, showing the presence of a properly folded protein. If not accessible then aggregation of the protein of interest has occurred. Wigley et al. developed self-assembling β-galactosidase fragments (α- and ω-) to monitor the solubility and folding of proteins in vivo. To test their method, they fused the α-fragment of β-galactosidase to Alzheimer Aβ (1-42) peptide, which aggregates readily and comprises the major component of amyloid plaques. When expressed with the ω-fragment no β-galactosidase activity was observed demonstrating aggregation, however when the α-fragment was fused to a mutant (F19P) of the Alzheimer Aβ (1-42) peptide that prevents insolubility, β-galactosidase activity was observed when expressed with the ω-fragment. Another example, of a protein tagging system involves
the self-assembly of split GFP fragments, split between amino acids 214 and 215 where the 1-214 fragment underwent three rounds of DNA shuffling to create a superfolded GFP fragment; improved solubility and increase complementation with fragment 215-230. Cabantous et al. expressed eighteen *Pyrobaculum aerophilum* test proteins with the C-terminal fragment of GFP and tested them *in vivo* and *in vitro* with the N-terminal fragment of GFP verifying a functional protein tagging system. These assays can facilitate efficient, high-throughput screening of promoters of protein folding or inhibitors of protein aggregation however there are drawbacks of each. The β-galactosidase tag can decrease solubility and the GFP system suffers from background scattering therefore expanding the split reporters available for protein tagging is desired.

**D. INHIBITION ASSAYS**

Inhibition assays include assay formats based on competitive inhibition, uncompetitive inhibition or mixed inhibition. In competitive inhibition, the antagonist binds to the active site of the free enzyme (receptor) only, whereas an uncompetitive inhibitor binds only to the enzyme-substrate (protein-ligand) complex. Mixed inhibition is a combination of competitive and uncompetitive inhibition where the antagonist binds either to the free receptor or the complex. One specific type of mixed inhibition is non-competitive inhibition where the antagonist binds to an allosteric site therefore preventing the substrate from binding in the active site. Regardless of the mechanism by which an inhibition assay works, they require that the antagonist inhibit the recognition component of the sensing system or inhibit the reporter itself. There are many inhibition assays that have been developed however herein only a few will be highlighted. Many of the earliest inhibition assays were based on radioactive labels using the law of mass action. Bradfield and Poland developed a competitive binding assay for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a known toxic halogenated aromatic hydrocarbon, and other ligands of the *Ah* receptor. This assay was developed such that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin competed with the radioligand, $[^{125}\text{I}]$-iodo-7,8-dibromodibenzo-*p*-dioxin for the *Ah* receptor yielding a detection limit of 10 pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and was proven to be a useful to screen related ligands of the *Ah* receptor. Sharma et al. developed a competitive binding assay for the screening of antagonist of the calcium
regulated protein calmodulin. In this case, an environmental sensitive fluorophore was chemically conjugated to calmodulin and when the hydrophobic probe, 2,6-anilinonaphthalene, bound to calmodulin fluorescence resonance energy transfer (FRET) occurred to excite the fluorophore. 2,6-Anilinonaphthalene can be displaced by analytes therefore, the screening system was developed so that the addition of a drug or peptide caused a competitive displacement of 2,6-anilinonaphthalene, consequently there was a decrease in the fluorescence of the fluorophore. This system was used to screen for antagonist of calmodulin and serves a general model for the development of other competitive binding assays for small molecules.\textsuperscript{147} In a more recent illustration, an inhibition assay for detecting mercury was developed. In this instance, mercury was quantified via the inhibition of the alcohol oxidase/horseradish peroxidase bi-enzymatic chemiluminescence reaction. The assay was found to have a linear range of 5-500 pg/mL mercury and this format was miniaturized yielding a detection limit of 1 pg/mL.\textsuperscript{148} In conclusion, inhibition assays are rapid, facile and cost effective methods of screening for antagonist and we foresee employing aequorin in inhibition assays.

**STATEMENT OF RESEARCH**

The overall goal of this dissertation is to develop biotechnology employing rational protein engineering which can then be applied to address specific problems. Specifically we plan to investigate the possibilities of developing functional split or truncated aequorin variants to be employed in innovative bioanalytical applications. Secondly, the variants developed will be analyzed to determine any changes in the bioluminescent properties of aequorin. Currently, all aequorin-based applications rely on the full-length apoprotein, whether the protein is a reporter via chemical conjugation or genetic fusion or is undergoing tuning to discover altered bioluminescent properties. The hypothesis of this work is that split aequorin can be used as a reporter in protein switch sensing systems and functional truncated aequorin fragments will be discovered. These newly discovered aequorin variants will increase the bioanalytical application of aequorin as well as increase the toolbox reporters for existing split and truncated systems.
Chapter two focuses on rationally designing the first split aequorin protein switch sensing system. The structure of aequorin was considered to design an insertion site in which another protein could be inserted. The rational design was followed leading to the creation of the first bioluminescent-based protein switch sensing system. The gene of the glucose-binding protein was inserted in between aequorin amino acid residues 47 and 48 and the resulting fusion protein was expressed and characterized. The fusion protein was used to develop a functional switch sensing system for the selective detection of glucose.

Chapter three includes the design of another protein switch sensing system. Here, the sulfate-binding protein was inserted into aequorin at the same rationally selected site as in chapter two. The resultant protein switch was characterized in terms of the bioluminescence characteristics and used to develop a sensing system for sulfate. The sensing system was able to detect sulfate in buffer as well as tap water, serum and simulated urine.

Chapter four began with developing an aequorin-based switch sensing system for hydroxylated polychlorinated biphenyls (OH-PCBs) using the regulator protein, HbpR, as the analyte recognition component. While characterizing the sensing system it was found that OH-PCBs drastically reduced the bioluminescence of aequorin. Because of this, we focused on developing an inhibition assay for the detection of OH-PCBs. This resulted in a unique sensing system that utilized aequorin alone as the recognition and reporting element. The bioluminescence characteristics of aequorin were characterized in the presence of a variety of OH-PCBs, and dose-response curves were generated for 15 different OH-PCBs. The sensing system was found to be useful to detect OH-PCBs in serum and tap water. Furthermore, mechanistic studies were performed leading to the belief that the basis of the sensing system is due to the OH-PCBs inhibiting the bioluminescence of aequorin by non-competitive inhibition.

In chapter five we tell our story of how a caspase-3 detection system motivated the idea of truncated aequorin. Originally, it was proposed to insert the caspase-3 recognition site into aequorin so that in the presence of caspase-3, aequorin was cleaved and bioluminescence was lost. When developing the assay it was found that aequorin
could be cleaved and retain bioluminescence. Therefore, this chapter is focused on proposing rationally designed truncated aequorin fragments that have the opportunity to be used as a reporter in protein complementation assays or protein tagging. Additionally, auto-luminescence fragments can be employed in multi-analyte detection or be better suited for *in vivo* applications. The chapter includes the current data including the characterization of the aequorin48-189 fragment that has a red shift in its emission peak.
CHAPTER TWO

A BIOLUMINESCENT MOLECULAR SWITCH FOR GLUCOSE

Introduction

Continuous discoveries in bioengineering and more efficient molecular biology methods have allowed scientists to create new designer biomolecules with unique and distinct properties. Protein switches with optical properties are an example of such designer biomolecules that in the presence of an environmental stimulus demonstrate an altered response manifested by an “on/off” signal. Such molecules can be employed in the development of nanosensors, creating allosteric enzymes, and as building blocks for the fabrication of functional nanobiomaterials with unique properties. Herein, we report a bioluminescent molecular switch created by insertion of glucose-binding protein (GBP) into the structure of the photoprotein aequorin (AEQ). In the presence of glucose, GBP undergoes a conformational change bringing the two segments of AEQ together, “turning on” bioluminescence and detecting glucose. This strategy provides a general approach to molecular switches given that proteins with ligand binding properties can be inserted into aequorin to create bioluminescent “on/off” nanosensors with potential for in vitro, as well as in vivo sensitive detection, and/or imaging applications.

Bionanotechnology has led to designer biomolecules tailored to perform nanoscale engineering functions.\textsuperscript{107,149} Such designer biomolecules include protein switches prepared by insertion of a nucleotide sequence coding for a desired function within the gene of a protein creating a chimeric protein with properties determined by both partners. Thus, two unrelated proteins can be fused to yield a protein switch with enhanced performance of one or both of the individual proteins.\textsuperscript{107} Optical protein switches using fluorescent proteins have been developed,\textsuperscript{103,104,107,108,150} however, no reports exist on the use of their bioluminescent counterparts, despite their superior detection capabilities. In that regard, we created a bioluminescence molecular switch by inserting the binding protein GBP into the bioluminescent protein AEQ in such a way
that glucose binding to GBP allosterically transduces AEQ, turning the switch “on” and generating bioluminescence emission (Figure 2.1).

Nature has provided a number of binding proteins with exquisite selectivity and sensitivity toward their ligand(s). Among these, the binding protein, GBP, was our choice as the model recognition protein for the proof-of-principle in the creation of bioluminescent molecular switches. GBP is a periplasmic binding protein capable of binding glucose with high selectivity. The protein consists of two globular domains linked by a flexible hinge region. Glucose binds to the hinge region causing a conformational change, which brings the two domains together engulfing the ligand. This conformational change has been previously employed in the development of sensitive, selective and reversible GBP-based fluorescence biosensors for glucose. It is well established that bioluminescence detection offers significant advantages over fluorescence, thus, employing bioluminescence in the creation of a molecular switch should result in highly sensitive systems with low detection limits. To that end, we selected the photoprotein aequorin as the bioluminescent generating partner for our glucose protein switch.

Materials and methods

Tris free base – Tris (hydroxymethyl) amino methane was purchased from Serva (Heidelberg, Germany). Glycine, sodium chloride, disodium ethylenediaminetetraacetate (EDTA), Luria-Bertani (LB) Agar, and LB Broth were purchased from Fischer Scientific (Fair Lawn, NJ). Albumin from bovine serum (BSA), ampicillin, ethidium bromide, protein A-alkaline phosphatase, sucrose, maltose, mannose, xylose, lactose, ribose, galactose and glucose were purchased from Sigma-Aldrich (St. Louis, MO). Pfu Ultra DNA polymerase and dNTPs were purchased from Agilent Technologies Stratagene Products Division (La Jolla, CA). Restriction enzymes were purchased from Promega (Madison, WI). Sodium Dodecyl Sulfate (SDS) was purchased from Curtin Mathesin Scientific, Inc. (Houston, TX). Coelenterazine was purchased from Gold Biotechnology, Inc. (St. Louis, MO). The Bradford protein assay kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Mini-prep kits and gel extraction kits were purchased
Figure 2.1. Schematic of the protein switch in it’s on/off mode.
from Qiagen (Valencia, CA). Gel code blue stain and 3,500 MWCO 3-12 mL Slide-A-Lyzer dialysis cassettes were purchased from Pierce (Rockford, IL). DEAE Sepharose Fast Flow was purchased from Amersham Bioscience (Uppsala, Sweden). Poros 50 HQ resin was purchased from PerSeptive Biosystems (Framingham, MA). Tris glycine SDS Page gels were purchased from Invitrogen (Carlsbad, CA). All chemicals were reagent grade or better and solutions were prepared using deionized reverse osmosis (Milli-Q Water Purification System, Millipore, Bedford, MA) water.

**Construction of Plasmid pGBPAEQ.** The apoaequorin cysteine-free mutant gene was used as the template for PCR. The following primers were designed to obtain the genes, which encode for aequorin amino acids 1-47 and aequorin amino acids 48-189:

(1)GTGGAATTCCAATGGTGAAACTGACCAGCGACTTCGACAACCCAAGATGG
(2)GCCAGAGAGCTCGAGCTGCAGGCTACCACCACCACCCGTGTTATTGATGACAATATCAG
(3)CACAGGCTTTTAGGGGACAGCTCCACCGTAGAGCTTTTCGGAAGCAGGC
(4)GTAGCCTGCAGCTCGAGCTCTCTGGCGGTGGCGGTTCTCTTGGAGCAACACCTGAGCAAG

Primers 1 and 2 were used to amplify the apoaequorin sequence coding for amino acids 1-47. Primer 1 introduced an *Eco*RI restriction site (underlined) on the 5’ end of the coding sequence, and primer 2 introduced a sequence coding for a six amino acid linker (SGGGGS) followed by restriction sites *Pst*I and *Sac*I (underlined) on the 3’ end. Primers 3 and 4 were used to amplify the apoaequorin sequence coding for residues 47-189. Primer 3 introduced a *Hind*III restriction site (underlined) on the 3’ end and primer 4 introduced the *Pst*I and *Sac*I restriction sites (underlined) followed by the SGGGGS linker on the 5’ end.

The apoAEQ1-47 and 48-189 gene sequences amplified above were then used as templates in an overlap PCR. Primers 1 and 3 from above were used in this PCR. This
resulted in a DNA sequence of apoAEQ containing the EcoR I site on the 5’ end and Hind III on the 3’ end with the linker, Pst I site, Sac I site, and linker between amino acids 47 and 48. The overlap PCR product was subcloned in the pCR2.1-TOPO vector, using the TOPO TA Cloning method by Invitrogen (Carlsbad, CA). Plasmid DNA was isolated using the Qiagen Mini Prep Kit (Valencia, CA).

The overlap apoAEQ DNA was digested with EcoR I and Hind III restriction enzymes. The plasmid, pIN4, which has a lpp-lac promoter and ompA leader sequence followed by a multiple cloning site was also digested with EcoR I and Hind III. Then, the digested aequorin DNA insert was ligated into pIN4 yielding pIN4-OLAEQ. The pIN4-OLAEQ vector was transformed into E. coli TOP10 cells, and the DNA was isolated. DNA sequencing was performed at the University of Kentucky Advanced Genetics Technology Center (AGTC) to confirm the DNA sequence.

To amplify GBP, the wild type GBP gene sequence, isolated from E. coli, was used as a template. The following primers were designed to obtain the GBP gene sequence:

(5.) GAGCTGCAGGCTGATACTCGCATTGGTGTAAC
(6.) CTTGAGCTCTTTCTTGCTGAGTTCAGCCAG

Primer 5 introduced a Pst I restriction site (underlined) on the 5’ end of the coding sequence and primer 6 introduced a Sac I restriction site (underlined) on the 3’ end. The DNA obtained by the PCR and plasmid pIN4-OLAEQ were digested with Pst I and Sac I restriction enzymes. The digested GBP fragment was ligated into pIN4-OLAEQ yielding pGBP1AEQ. DNA sequencing was performed to verify the gene sequence encoding for AEQ1-47-linker-GBP-linker-AEQ 48-189 fusion protein.

Expression and Purification of GBP-AEQ fusion protein. The pGBP1AEQ vector was transformed into chemically competent E. coli Top10 cells. The bacterial cells were grown overnight at 37 °C in 5 mL of Luria Bertani broth containing 100µg/mL
ampicillin. This culture was used to inoculate 500 mL of broth containing 100 µg/ml ampicillin, and this was grown at 37 °C. When the culture reached an OD₆₀₀ of 0.4, IPTG was added to 1 mM final concentration, and the bacteria were left to grow overnight. The cells were harvested by centrifugation at 22,100 x g at 4 °C. The pellet was resuspended in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA buffer and sonicated on ice using 10-s bursts followed by 10-s rest for 5 min total using a Fischer Scientific 550 Sonic Dismembrator (Pittsburg, PA). The suspension was centrifuged at 22,100 x g at 4 °C for 15 min to obtain the pellet containing the inclusion bodies. The pellet was resuspended in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl buffer followed by centrifugation at 22,100 x g at 4 °C. The pellet was washed once with 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 1 % (v/v) Triton X-100 buffer, and then with 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 5 mM CaCl₂ buffer with centrifugation at 22,100 x g, at 4 °C, for 10 min between washes to pellet the inclusion bodies. The pellet was resuspended in 30 mM Tris/Cl, pH 7.5, 2 mM EDTA, 6 M urea buffer and left to rotate at 4 °C overnight to denature the fusion protein. Urea is a chaotropic agent and high concentrations of urea will disrupt the hydrophobic interactions of a protein thus denaturing the protein. After 24 hours, the denatured inclusion bodies were centrifuged at 22,100 x g at 4 °C for 20 min to obtain the supernatant containing the denatured fusion protein.

The denatured protein was purified using a BioCAD Sprint Perfusion chromatography system of PerSeptive Biosystems (Framingham, MA) with a DEAE Sepharose-ion exchange column. The column was equilibrated with 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 6 M urea buffer. The supernatant containing the protein was loaded on the column, and the column was washed with 3 column volumes of the equilibration buffer. The protein was eluted using a gradient of 0 % (no elution buffer) to 50 % elution buffer (30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 6 M urea, 1 M NaCl) over 10 column volumes. Five milliliters fractions were collected. An SDS-PAGE was run to verify the fractions containing the denatured fusion protein, which were pooled together. Excess salt was removed from the combined fractions by buffer exchange with 1 L of 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 6 M urea using a hollow fiber filter and a peristaltic pump. The urea must be removed from the denatured protein to allow the protein to
refold into its active conformation. To do this, the denatured protein was renatured and purified using the BioCAD Sprint Perfusion chromatography system with a Poros 50 HQ ion exchange column in the absence of urea. The column was equilibrated with 30 mM Tris/HCl, pH 7.5, 2 mM EDTA buffer. The fractions containing the denatured protein were loaded on the column and the column was washed with 3 column volumes of the equilibration buffer. The fusion protein was eluted using a gradient of 0 % to 50 % elution buffer (30 mM Tris/Cl, pH 7.5, 2 mM EDTA, 1 M NaCl) over 10 column volumes. Five milliliter fractions were collected. An SDS-PAGE gel determined the purity of the fractions. The fractions containing the pure renatured apofusion protein were pooled together. The protein concentration was determined by the Bradford protein assay, with BSA as a standard.

**Bioluminescence Emission Study.** A 3 times molar excess of coelenterazine was added to the apoaequorin-GBP fusion protein, and the mixture was left at 4 °C for 18 h. Ten microliters of GBP-AEQ fusion protein (6.14 x 10\(^{-7}\) M) was assayed in an Optocomp I Test Tube Luminometer (MGM Instruments, Inc., Hamden, CT). Bioluminescence was triggered by injecting 50µL of 100mM Tris/Cl pH 7.5 100mM CaCl\(_2\) buffer. The bioluminescence signal was integrated for 6-s.

**Bioluminescence Emission Spectra.** A 3 times molar excess of coelenterazine was added to the apoaequorin-GBP fusion protein (6.14 x 10\(^{-7}\) M), and the mixture was left at 4 °C for 18 h. Also the stock solution of GBP-AEQ fusion protein (6.14 x 10\(^{-7}\) M) was serial diluted in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA buffer. Then, 100 µL of each dilution was added to three 1.5 mL polypropylene microcentrifuge tubes. A final concentration of 1 µg/mL coelenterazine was added to each concentration of the apoaequorin-GBP fusion protein and this mixture was left at 4 °C for 18 h. For each concentration, a volume of 10 µL of GBP-AEQ fusion protein was added to a Microtiter plate, and bioluminescence was triggered by injecting 50 µL of 100 mM Tris/Cl, pH 7.5, 100 mM CaCl\(_2\) buffer. The bioluminescence signal was between 400 and 1200 nm was determined on a Luminoskan Ascent CCD camera luminometer (Thermo LabSystems, Waltham, MA).
**Bioluminescence Half Life Study.** A 3 times molar excess of coelenterazine was added to the apoaequorin-GBP fusion protein and this mixture was left at 4 °C for 18 h. A volume of 10 µL of the GBP-AEQ fusion protein (6.14 x 10^{-7} M) was added to a disposable glass tube. Bioluminescence was triggered by injecting 50 µL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl₂ buffer, and bioluminescence was measured on the Optocomp I luminometer. The bioluminescence signal was collected for 6-s and the half-life was calculated using the one phase exponential decay kinetics equation in GraphPad Prism. The same procedure was performed for the native protein using a concentration of 1.13 x 10^{-9} M.

**GBP-AEQ Characterization.** The stock solution of apoaequorin-GBP fusion protein (6.14 x 10^{-7} M) was diluted to 5.0 x 10^{-8} M in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA buffer and coelenterazine was added to a final concentration of 1 µg/mL. Immediately, 90 µL was added to separate 1.5 mL polypropylene microcentrifuge tubes. A 1 M stock solution of glucose was serial diluted in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA. Ten microliters of each glucose concentration was added to separate tubes containing the fusion protein. The tubes were left to charge overnight at 4 °C. Ten microliters from each tube was added to a disposable glass tube. Bioluminescence was triggered by injecting 50 µL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl₂ buffer, and bioluminescence was measured in the Optocomp I luminometer. The data was plotted as total intensity (RLU) versus log[glucose]. The data was fitted using the sigmoidal dose-response (variable slope) equation of GraphPad Prism 4.

To determine the selectivity of the GBP-AEQ, the stock solution of apoaequorin-GBP fusion protein (6.14 x 10^{-7} M) was diluted to 5.0 x 10^{-8} M in 30 mM Tris/HCl pH 7.5 2 mM EDTA buffer and coelenterazine was added to a final concentration of 1µg/mL. Immediately, 90 µL was added to separate 1.5 mL polypropylene microcentrifuge tubes. One hundred millimolar solutions of galactose, ribose, sucrose, maltose, mannose, xylose, and lactose were prepared in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA buffer. Ten microliters of each sugar was added to separate tubes containing the fusion protein. The tubes were left to charge overnight at 4 °C. Ten microliters from each
tube was added to a disposable glass tube. Bioluminescence was triggered by injecting 50 µL of 100 mM Tris/HCl pH 7.5 100 mM CaCl₂ buffer, and bioluminescence was measure in the Optocomp I luminometer.

**Circular Dichroism (CD).** The concentration of each protein was determined by their absorbance at the 280 nm. Aequorin and GBP-AEQ without glucose were charged overnight at 4 °C with coelenterazine to a final concentration of 1µg/mL. Glucose was added to GBP to a final concentration of 10 mM and kept overnight at 4 °C. For GBP-AEQ with glucose, 10 mM glucose and 1 µg/mL coelenterazine were added to the protein and kept overnight at 4 °C. The CD spectra were collected with a JASCO J-810 spectrometer (JASCO Ltd. UK) with a 1 nm bandwidth using a 0.1 cm pathlength cuvette for the far UV scans, and a 1.0 cm pathlength cuvette for the near UV scans. The protein samples were dialyzed with a low salt buffer (5 mM phosphate buffer, pH 7.0, 2mM EDTA) before the CD measurement. The wavelength scan was performed at 20 °C, and corrected for the blank. For aequorin and GBP-AEQ without glucose, the blank was composed of the low salt buffer and coelenterazine. For GBP, the blank was composed of the low salt buffer and 10 mM glucose, and for GBP-AEQ with glucose, the blank was composed of the low salt buffer, coelenterazine, and 10 mM glucose.

**Results and discussion**

Aequorin consists of an apoprotein and a chromophore that resides in a hydrophobic binding pocket. In the presence of molecular oxygen and Ca²⁺ AEO undergoes a conformational change that leads to the oxidation of the chromophore, coelenterazine, to coelenteramide and the release of CO₂ with a concomitant emission of light at 469 nm. ¹⁵⁵ AEO has three calcium binding EF-hands²⁴ (EF-hand I, III, and IV), and three triads each consisting of tryptophan, tyrosine and histidine, which are involved in holding coelenterazine in the active site (Figure 2.2). ¹⁵⁶ Thus, these EF-hands and triads are essential for the maximum activity of the protein, and their disruption could lead to loss of protein stability and bioluminescence. The creation of our molecular switch involved a rational strategy where the structure of AEQ was split into two
Figure 2.2. Three-dimensional structure of aequorin with coelenterazine (1EJ3). EF-Hand I is in blue. EF-hand III is in green. EF-hand IV is in red. The arrow points out the insertion site. Visualized using Discovery Studio Visualizer 2.5.5.
fragments, and GBP was inserted in between those fragments. In the resulting hybrid protein the split AEQ fragments are too far apart to re-assemble, and thus, the molecular switch is “off”. However, the presence of glucose induces a conformational change in GBP that allows for the AEQ fragments to come together and freely re-form into one bioluminescent active entity, turning the switch “on”. A comparison of the near UV circular dichroism (CD) spectra of GBP-AEQ in the presence or absence of glucose has shown that the protein tertiary structure rearranged upon binding glucose, as indicated by the peak shift at approximately 290 nm as well as the difference in spectra between 280 and 260 nm (Figure 2.3).

The three-dimensional structure of AEQ demonstrates an exterior flexible loop between EF-hand I and EF-hand II (Figure 2.2) into which we hypothesized that a large sequence might be inserted without destroying the activity of AEQ. Thus, GBP was inserted between amino acids 47 and 48 (Figure 2.4). Indeed, neither the calcium binding sites nor the amino acids that interact with coelenterazine were disrupted. The specific activity of the hybrid protein was found to be $7.5 \times 10^9$ RLU (Relative Light Units)/mg; in comparison that of parent aequorin was $3.65 \times 10^{12}$ RLU/mg. Thus, while the insertion of GBP into the AEQ structure lowered the activity of the protein, due to aequorin being in a less active conformation, it is still able to allow for the reunion of the two spliced fragments of AEQ, maintaining the protein recognition of GBP for glucose, as well as the emission of significant bioluminescence. Thus, the ligand bound to the fusion protein leads to a more normal active conformation of aequorin. The $\lambda_{\text{max}}$ of GBP-AEQ was found to be 471 nm, which is essentially the same as the $\lambda_{\text{max}}$ of 472 nm of the parent aequorin. In addition, the GBP-AEQ fusion protein demonstrated the same flash-type kinetics as the parent aequorin. The half-life of the GBP-AEQ fusion protein charged with the native coelenterazine chromophore was found to be 2.16 s, which is considerably different than the 0.70 s half-life of the parent aequorin. The longer half-life could be attributed to GBP inserted in between the split halves of AEQ. As shown by the specific activity, the insertion of GBP reduces the luminescence activity to some extent; however, it does not considerably affect the spectral properties of photoprotein aequorin. To the best of our knowledge, this is the first time that AEQ has been split into
Figure 2.3. Near UV CD spectra. The spectra were collected with a JASCO J-810 spectrometer (JASCO Ltd. UK) with 1 nm bandwidth and using a 1.0 cm pathlength cuvette. The wavelength scans were corrected for the blank and converted into molar ellipticity.
Figure 2.4. An illustration of the three-dimensional structure of the GBP-AEQ molecular switch in solution. Composed of glucose binding protein (GBP) and split aequorin. Visualized using Discovery Studio Visualizer 2.5.5.
fragments, and that the AEQ fragments have demonstrated “on” bioluminescence upon a molecular recognition event.

In the presence of glucose, the protein switch demonstrated an increase in bioluminescence intensity proportional to the levels of glucose, demonstrating glucose detection down to $1.0 \times 10^{-7}$ M (Figure 2.5). The switch is “on” and “off” between $1.0 \times 10^{-7}$ to $1.0 \times 10^{-2}$ M concentrations of glucose, which encompasses the normal, hypoglycemic and hyperglycemic glucose levels in blood, from 2 to 20 mM glucose. Furthermore, the sugars not known to bind to GBP had no effect on the molecular switch, demonstrating that GBP confers its high selectivity pattern for glucose to the GBP-AEQ switch (Figure 2.6). Thus, the molecular switch could be useful in the clinical detection of glucose.

In summary, we have demonstrated that genetic manipulation to create a hybrid protein of two unrelated proteins, namely AEQ and GBP, can result in a functional bioluminescent protein switch whose “on/off mode” is tuned by the recognition of GBP toward glucose. This is the first time that aequorin has been rationally split into two fragments that upon a molecular recognition event come together and emit bioluminescence forming the basis for the creation of the first bioluminescent molecular switch. More significantly, this work demonstrates the generation of a whole new family of genetically encoded bioluminescent protein switches by introduction of a binding protein into the structure of the spliced AEQ. A variety of hinge-motion binding proteins, such as periplasmic binding proteins, enzymes, transcriptional regulators or messenger proteins could be employed in such protein switches. It is envisioned that these newly created protein chimeras with distinct properties and functions could be employed in a variety of applications, such as in highly sensitive and selective biosensing nanosystems, or as functional building blocks for the fabrication of “bottom-up” smart bionanomaterials with unique characteristics for the incorporation into nanodevices. Further, this work provides useful insight into the areas of genetically engineered chimeric proteins, allowing for the design and production of a series of other unique “designer” biomolecules.
Figure 2.5. Glucose response. The performance of the molecular switch to sensing glucose was evaluated by employing a concentration of $5.0 \times 10^{-8}$ M of hybrid protein. The data points are an average of three measurements ± 1 standard deviation.
Figure 2.6. Light intensity in the presence of 10mM of the ligand. The relative light units (RLUs) without a ligand were subtracted from the RLUs with the each ligand. The data points are an average of three measurements ± 1 standard deviation.
CHAPTER THREE

A PROTEIN SWITCH SENSING SYSTEM FOR THE QUANTIFICATION OF SULFATE

Genetic engineering is a commonly used tool for the creation of new biomolecules with unique and distinct properties. Genetically engineered protein switches, which are able to respond to an environmental stimulus with a signal of an “on/off” nature, are among such designer molecules. Protein-based molecular switches are potentially useful for the development of biosensing systems, as therapeutic agents, and diagnostic tools.\(^{157,158}\) Protein switches incorporating optical detection have been employed to create sensing systems with tailored characteristics.\(^{101,103,104,107,108,159}\) A protein switch consists of two unrelated proteins that are linked together such that changes in one protein domain will modulate the function of the second protein domain. For the optical-based protein switch sensing system considered here, the proteins work together simulating a light switch; one protein will bind to an analyte, thus undergoing a change in conformation that propagates to a second protein and generates an alteration in its activity. This causes the switch to turn “on and off” allowing for the detection of the analyte. To that end, we have designed a bioluminescence-based protein switch by coupling the bioluminescent protein aequorin (AEQ) with the sulfate-binding protein (SBP), yielding a sensing system for sulfate.

Aequorin is a calcium dependent photoprotein originally isolated from the jellyfish *Aequorea victoria*.\(^{160,161}\) It consists of the 189 amino acid apoprotein, apo-AEQ, and the organic chromophore coelenterazine (Figure 3.1(a)). The structure of AEQ contains two pairs of EF-hands and three triads each containing the amino acids tryptophan, tyrosine and histidine, involved in holding the coelenterazine in the active site of the protein through different types of bonding interactions.\(^{156}\) Three of the EF-hands have canonical Ca\(^{2+}\)-binding sequences,\(^{19}\) while all four EF-hands fold together creating a hydrophobic core within the structure of the protein, which interacts and
Figure 3.1. (a) The 3-D crystal structure of aequorin. Coelenterazine is non-covalently bound in the hydrophobic pocket. Protein data bank structure 1EJ3. (b) The 3-D crystal structure of the sulfate-binding protein with sulfate bound in the hinge region. Protein data bank structure 1SBP. Images visualized in Accelrys Discovery Studio Visualizer 2.5.
stabilizes coelenterazine. In the presence of molecular oxygen and calcium, AEQ undergoes a conformational change that leads to the oxidation of coelenterazine to coelenteramide, and the release of CO\textsubscript{2} with a concomitant emission of light at 470 nm\textsuperscript{155}. Employing AEQ as the reporter in a protein switch sensing system has an advantage over its fluorescent counterparts due to lack of background scattering, a typical drawback of fluorescence-based methods. As a result, AEQ can be detected at very low levels, and when used as a reporter it allows for detection down to attomole levels; therefore, the detection limits of the sensing system should be limited by the receptor domain as opposed to the reporter domain.

The sulfate-binding protein, first identified from \textit{Salmonella typhimurium} in 1965, is a periplasmic binding protein found in Gram-negative bacteria. SBP is a 310 amino acid residue protein with a molecular weight of 35 kDa\textsuperscript{162} (Figure 3.1(b)) that binds to tetrahedral, fully ionized, oxyacid dianions with high selectivity\textsuperscript{163}. The crystal structure demonstrates that SBP is ellipsoidal in shape with two globular domains linked by a flexible hinge region that consists of three peptide strands. Sulfate is bound, via hydrogen bonds and van der Waals forces, deep within the hinge region\textsuperscript{115,164}. Moreover, the binding of sulfate causes a conformational change in the hinge region of the protein\textsuperscript{165}. This conformational change is the key to detect binding of the sulfate ligand to the protein, and forms the basis for the development of this protein switch sensing system for sulfate.

Sulfate is an important oxoanion that participates in both biological and environmental events and pathways. Sulfate is a metabolite of the trans-sulfuration pathway and plays a critical role in heavy metal detoxification in invertebrate animals\textsuperscript{166}. Thus, the detection of sulfate is important to both, the medical and environmental fields. An interesting observation that merits further examination relates to recent studies showing that children with autism have reduced levels of sulfate in their blood\textsuperscript{167,168}, but elevated levels of sulfate in their urine\textsuperscript{169}. The decrease in blood sulfate levels in autism patients is shown to be associated with impaired sulfation\textsuperscript{170}, which can result in
decreased detoxification. On a different, yet perhaps as relevant of an observation, sulfate may be an environmental risk and there are concerns about its presence in drinking water. Sulfate occurs naturally in drinking water and reports show that diarrhea is associated with high levels of sulfate being ingested. Accordingly, efficient methods of quantification of sulfate are desirable.

Traditional methods for the quantification of sulfate include gravimetry, turbidimetry, colorimetry and ion chromatography. There are many other methods, such as, sulfate selective electrodes, atomic absorption spectrometry, electrochemistry and protein-based sensing systems, to name a few. While these methods are widely employed and are very valuable they suffer from limitations that, in some instances, impede their use in particular applications. For example, chromatography methods are highly complex and require expensive instrumentation, therefore, they are not ideal for on-site detection. Herein, we have developed an optical-based protein switch sensing system for the detection of sulfate, which is rapid and amenable to miniaturization. Specifically, we have employed genetic engineering to insert the sensing protein, SBP, within the sequence of the reporting protein, AEQ, in such a manner that when sulfate binds to SBP, its conformational change is allosterically propagated to AEQ, triggering emission of bioluminescence of AEQ in a dose-dependent fashion. Fusion protein switch sensing systems have an advantage over other protein-based sensing systems due to the fact that no chemical coupling or separation steps are required, thus facilitating preparation of the sensing system. The detection limits for sulfate and the working ranges obtained with this molecular switch are within the levels needed for measurement of sulfate in biological and environmental samples, $3.0 \times 10^{-4} \text{ M}$ and $2.60 \times 10^{-3} \text{ M}$ for human serum and drinking water, respectively; therefore, our sensing system provides a viable alternative method for the detection of sulfate.

Materials and Methods

Materials. Tris free base – Tris (hydroxymethyl) amino methane was purchased from Serva (Heidelberg, Germany). Glycine, sodium chloride, disodium ethylenediaminetetraacetate (EDTA), Luria-Bertani (LB) Agar and LB Broth were
purchased from Fischer Scientific (Fair Lawn, NJ). Sodium sulfate was purchased from J. T. Baker (Phillipsburg, NJ). Albumin from bovine serum (BSA), lanthanum chloride (LaCl₃), kanamycin monosulfate, ethidium bromide, dimethyl sulfoxide (DMSO) anhydrous, potassium chloride, sodium selenate, sodium nitrate, sodium arsenate, sodium perchlorate, protein A-alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate disodium salt and nitrotetrazolium blue chloride were purchased from Sigma-Aldrich (St. Louis, MO). Pfu Ultra DNA polymerase and dNTPs were purchased from Agilent Technologies Stratagene Products Division (La Jolla, CA). Restriction enzymes were purchased from Promega (Madison, WI). Sodium dodecyl sulfate (SDS) was purchased from Curtin Mathesin Scientific, Inc. (Houston, TX). Coelenterazine was purchased from Gold Biotechnology, Inc. (St. Louis, MO). The Bradford protein assay kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). DNA isolation kits, gel extraction kits and Ni-NTA resin were purchased from Qiagen (Valencia, CA). The plasmid, pET-28(a)⁺, and BL21(DE3) chemically competent cells were purchased from Novagen (Madison, WI). Gel code blue stain and 3,500 Da molecular weight cut off (MWCO) 3-12 mL Slide-A-Lyzer dialysis cassettes were purchased from Pierce (Rockford, IL). DEAE Sepharose Fast Flow was purchased from Amersham Bioscience (Uppsala, Sweden). Rabbit polyclonal anti-AEQ primary antibody (Catalogue # ab9096) was purchased from Abcam (Cambridge, MA). Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and polyvinylidene fluoride (PVDF) membranes were purchased from Invitrogen (Carlsbad, CA). All chemicals were reagent grade or better and solutions were prepared using deionized reverse osmosis (Milli-Q Water Purification System, Millipore, Bedford, MA) water.

**Construction of Plasmid pSBPAEQ.** The apoaequorin cysteine-free mutant gene was used as the template for the polymerase chain reaction (PCR). The following primers were designed to obtain the genes, which encode for apoaequorin amino acids 1-47 and apoaequorin amino acids 48-189:

(1) GTGGCTAGCATGTTGAAAATGACCAGCGACTTGGACAAAACAGATG

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Primers 1 and 2 were used to amplify the apoaequorin sequence coding for amino acids 1-47. Primer 1 introduced an \textit{Nhe} I restriction site (underlined) on the 5′ end of the coding sequence, and primer 2 introduced a sequence coding for a six amino acid linker (SGGGGS) followed by restriction sites \textit{Pst} I and \textit{Sac} I (underlined) on the 3′ end. Primers 3 and 4 were used to amplify the apoaequorin sequence coding for residues 48-189. Primer 3 introduced a \textit{Hind} III restriction site (underlined) on the 3′ end and primer 4 introduced the \textit{Pst} I and \textit{Sac} I restriction sites (underlined) followed by the SGGGGS linker on the 5′ end. The PCRs were performed using the Pfü Ultra polymerase employing 30 cycles with denaturing, annealing and elongating conditions of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 2 minutes, respectively. The AEQ1-47 and 48-189 gene sequences amplified above were then used as templates in an overlap PCR. Primers 1 and 3 from above were used in this PCR. This resulted in a DNA sequence of apo-AEQ containing the \textit{Nhe} I site on the 5′ end and \textit{Hind} III on the 3′ end with the linker, \textit{Pst} I site, \textit{Sac} I site, and linker between amino acids 47 and 48. The overlap PCR product was subcloned in the pCR2.1-TOPO vector, using the TOPO TA Cloning method by Invitrogen (Carlsbad, CA). Plasmid DNA was isolated using the Qiagen DNA Isolation Kit (Valencia, CA). The overlap apo-AEQ DNA was digested with \textit{Nhe} I and \textit{Hind} III restriction enzymes. The plasmid, pET-28a(+), was also digested with \textit{Nhe} I and \textit{Hind} III and the digested apoaequorin DNA insert was ligated into pET-28a(+) yielding pET-28a(+)-OLAEQ. The pET-28a(+)-OLAEQ vector was transformed into chemically competent \textit{E. coli} BL21(DE3) cells, and the DNA was isolated.

To amplify SBP, the wild type SBP gene sequence, isolated from \textit{E. coli}, was used as a template. The following primers were designed to obtain the SBP gene sequence:

\textbf{(5)} GAGCTGCAGAAGGATATTCAGCTTCTTAACGT
Primer 5 introduced a *Pst* I restriction site (underlined) on the 5’ end of the coding sequence and primer 6 introduced a *Sac* I restriction site (underlined) on the 3’ end. The DNA obtained by the PCR and plasmid pET-28a(+)-OLAEQ were digested with *Pst* I and *Sac* I restriction enzymes. The digested SBP fragment was ligated into pET-28a(+)-OLAEQ yielding pSBPAEQ. DNA sequencing was performed to verify the gene sequence encoding for apo-AEQ1-47-linker-SBP-linker-apo-AEQ48-189 hybrid protein. DNA sequencing was performed at the University of Kentucky Advanced Genetics Technology Center (AGTC) to confirm the correct, in frame, DNA sequence.

**Expression and Purification of SBP-apo-AEQ hybrid protein.** The pSBPAEQ vector was transformed into chemically competent *E. coli* BL21(DE3) cells. The bacterial cells were grown overnight at 37 °C x 250 rpm in 5 mL of Luria Bertani broth containing 30 µg/mL kanamycin. This culture was used to inoculate 500 mL of broth containing 30 µg/mL kanamycin, and this was grown at 37 °C x 250 rpm. When the culture reached an OD₆₀₀ of 0.2 the culture was moved to a room temperature shaker (250 rpm). When the culture reached an OD₆₀₀ of 0.4, IPTG was added to 0.5 mM final concentration, and the bacteria were left to grow overnight. The cells were harvested by centrifugation at 22,100 x g at 4 °C for 15 minutes. The pellet was resuspended in 5 mL of 10 mM Tris/HCl, pH 8.0, 100 mM NaH₂PO₄, 8 M urea buffer for every gram of pellet, and placed on a room temperature orbital shaker at 200 rpm for one hour. Then the resuspended pellet was sonicated on ice using 10 second bursts followed by 10 second rests for 5 min total using a Fischer Scientific 550 Sonic Dismembrator (Pittsburg, PA). The suspension was centrifuged at 11,100 x g at 4 °C for 30 min to obtain the supernatant containing the inclusion bodies. For every 4 mL of supernatant 1 mL Ni-NTA Agarose was added and placed on room temperature orbital shaker at 200 rpm for one hour. The supernatant containing the agarose beads was loaded into gravity flow column and flow through was collected. The Ni-NTA agarose was washed with 2 x 4 mL of 10 mM Tris/HCl, pH 6.3, 100 mM NaH₂PO₄, 8 M urea buffer; and the wash was collected. The resin was then washed with 4 x 0.5 mL fractions of 10 mM Tris/HCl, pH 5.9, 100 mM NaH₂PO₄, 8 M
urea buffer; the wash fractions were collected. The protein was eluted with 8 x 1.5 mL fractions of 10 mM Tris/HCl, pH 4.5, 100 mM NaH$_2$PO$_4$, 8 M urea buffer. An SDS-PAGE gel was run to verify the fractions containing the denatured hybrid apoprotein. The elution fractions containing the most hybrid apoprotein were combined and dialyzed overnight against 2 L of 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 4 M urea buffer using 3500 MWCO Pierce dialysis cassettes. The dialyzed apoprotein was further purified using a BioCAD Sprint Perfusion chromatography system (PerSeptive Biosystems, Framingham, MA) with a DEAE Sepharose Fast Flow-ion exchange column (10 mm dia x 70 mm). The column was equilibrated with 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 4 M urea buffer. The apoprotein was loaded onto the column, and the column was washed with three column volumes of the equilibration buffer. The hybrid apoprotein was eluted using a gradient of 0 % (no elution buffer) to 50 % elution buffer (30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 1 M NaCl) over ten column volumes. Five milliliters fractions were collected. An SDS-PAGE gel was run to verify the fractions containing the apoprotein. The fractions containing the most hybrid apoprotein were combined and dialyzed in a step-wise manner to renature the hybrid apoprotein. The procedure was as follows: the hybrid apoprotein was dialyzed in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 2 M urea buffer, dialyzed in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 1 M urea buffer, dialyzed in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 0.5 M urea buffer, dialyzed in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 0.25 M urea buffer. Finally, the apoprotein was dialyzed into 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer. Each dialysis step was performed using Pierce 3500 MWCO dialysis cassettes in 2 L of the appropriate buffer at 4 °C for 24 hours. A western blot determined the presence of the apoprotein, and the concentration was determined by the Bradford protein assay, using BSA as a standard.

**Western Blot.** A native SDS-Page gel was run using standard protocols with the Benchmark pre-stained ladder (Invitrogen). The apoproteins were transferred onto a PVDF membrane in 1X Novex Tris Glycine Transfer buffer (Invitrogen) with 20% methanol for 1 hour at 25 volts. The membrane was blocked with blocking buffer, 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10% (w/v) nonfat dry milk, for 30 minutes at room temperature while shaking gently. The blocking buffer was removed and the primary
antibody (Rabbit polyclonal to AEQ) diluted 1:1000 in the blocking solution was added to the membrane. The membrane was incubated in the primary antibody solution for one hour while gently shaking at room temperature and then removed. The membrane was washed three times, for ten minutes, with 15 ml of 20 mM Tris/HCl, pH 7.5, 150 mM NaCl buffer. The secondary antibody, protein A-alkaline phosphatase, diluted 1:2000 in the blocking solution, was added to the membrane and to the membrane was incubated at room temperature for one hour while shaking gently. Then, the secondary antibody was removed and the membrane was washed three times, for ten minutes, with 15 mL of 20 mM Tris/HCl, pH 7.5, 150 mM NaCl buffer. The membrane was washed a final time with 15 mL of 100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂ buffer at room temperature for ten minutes. The staining solution, 20 mL of 100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂ buffer containing 100 µL aliquots of 20 mg/mL 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) and 50 mg/mL nitrotetrazolium blue chloride (NTB) in dimethyl formamide was added to the PVDF membrane and incubated with gentle shaking at room temperature until the blot was developed. The staining reaction was stopped by washing the membrane with D. I. water and left to dry.

**Bioluminescence Emission Study.** A three times molar excess of native coelenterazine was added to the SBP-apo-AEQ hybrid protein and the mixture was incubated at 4 °C for 18 h to form SBP-AEQ. The bioluminescence activity of SBP-AEQ hybrid protein was measured in an Optocomp I Test Tube Luminometer (MGM Instruments, Inc., Hamden, CT) by mixing 10 µL of SBP-AEQ hybrid protein with 50 µL of triggering buffer (100 mM Tris/HCl, pH 7.5, 100 mM LaCl₃). The bioluminescence signal was integrated for 6-s, and intensity was recorded as relative light units (RLUs).

**Dilution Study for SBP-AEQ.** The stock solution of SBP-apo-AEQ protein was serially diluted in 30 mM Tris/HCl, pH7.5, 10 mM EDTA, 0.01% BSA buffer. In a polystyrene microtiter plate, 100 µL of each concentration of diluted protein, in triplicates, was incubated with a three times molar excess of native coelenterazine overnight at 4 °C. Then the bioluminescence was triggered by injecting triggering buffer and the intensity of
the bioluminescence was integrated for 6-s on a FLUOstar Optima luminometer (BMG Labtech, Cary, NC).

**Time Study.** The stock solution of SBP-apo-AEQ was diluted to 1.0 x 10⁻⁷ M in 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA. In order to form the active SBP-AEQ, coelenterazine was added to the aforementioned solution of to a final concentration of 1 µg/mL and incubated at 4 °C for 16 hours. A 3.16 x 10⁻³ M stock solution of sodium sulfate was made in 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer. Twenty microliters of this stock sulfate solution was added to 80 µL of the hybrid protein. Sulfate-free control measurements were performed by mixing 20 µL of 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer with 80 µL of the hybrid protein. Each measurement was performed in triplicates. After 5, 10, 15, 30 and 60 minutes of incubation at room temperature, 5 µL of the solutions was checked for bioluminescence activity by injecting 100 µL of the triggering buffer. The bioluminescence signal was integrated for 6-s on a FLUOstar Optima luminometer (BMG Labtech, Cary, NC).

**Bioluminescence Emission Spectra.** Three times molar excess of coelenterazine was mixed with SBP-apo-AEQ hybrid protein and the mixture was incubated at 4 °C for 18 h. The emission maximum of the bioluminescence reaction was determined in the presence and absence of sulfate ions. For samples containing sulfate, 80 µL of SBP-AEQ was added to three separate wells of a microtiter plate and 20 µL of 3.16 x 10⁻³ M sodium sulfate was added. For control samples without sulfate, 80 µl of SBP-AEQ was added to three separate wells of a microtiter plate and 20 µL of 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer was added. After 15 minutes of incubation, bioluminescence was triggered by injecting 100 µL of triggering buffer. The bioluminescence signal was collected on a Spectro System Spectrographic Luminometer custom built by Sciencewares, Inc. (Falmouth, MA) between 400 and 700 nm.

**Bioluminescence Half Life Study.** A three times molar excess of coelenterazine was mixed with SBP-apo-AEQ hybrid protein and incubated at 4 °C for 18 h. The half-life of the bioluminescence emission of SBP-AEQ was determined both in the presence and
absence of sulfate ions. For samples containing sulfate ions, 80 µL of SBP-AEQ was added to three separate wells of a microtiter plate and 20 µl of 3.16 x 10^{-3} M sodium sulfate was added. For samples without sulfate, 80 µL of SBP-AEQ was added to three separate wells of a microtiter plate and 20 µl of 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer was added. After 15 minutes of incubation at room temperature, bioluminescence was triggered by injecting 100 µL of triggering buffer, and bioluminescence emission intensity was measured on the FLUOstar Optima luminometer. The bioluminescence signal was collected for 6-s at 100 µsec intervals and the half-life was calculated by fitting the data points to one phase exponential decay kinetics equation in GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

**Circular Dichroism (CD).** The stock solution of SBP-apo-AEQ was dialyzed overnight against 2 L of a low salt buffer (5 mM phosphate, pH 7.0, 10 mM EDTA). The dialyzed protein was concentrated by centrifugation for 30 minutes at 4000 x g in Amicon Ultra centrifugal filters, 10,000 MWCO (Millipore, Billerica, MA). The concentration of protein was determined by their absorbance at the 280 nm. The concentrated solution of SBP-apo-AEQ hybrid protein was incubated overnight at 4 °C with coelenterazine to a final concentration of 1µg/mL. CD measurements were performed in the presence and absence of sulfate. For samples containing sulfate ions, 400 µL of 3.16 x 10^{-3} M sodium sulfate, prepared in low salt buffer, was added to 1600 µL of SBP-AEQ solution. For control samples without sulfate ions, 400 µL of 5 mM phosphate, pH 7.0, 10 mM EDTA buffer was added to 1600 µL of SBP-AEQ. The CD spectra were collected by using a JASCO J-810 spectrometer (JASCO Ltd., UK) with a 1 nm bandwidth using 1.0 cm pathlength cuvettes for the near UV scans. The wavelength scan was performed at 20 °C, and corrected for the blank. For SBP-AEQ without sulfate, the blank was composed of the low salt buffer and coelenterazine. For SBP-AEQ with sulfate, the blank was composed of the low salt buffer, coelenterazine and 3.16 x 10^{-3} M sodium sulfate.

**SBP-AEQ Response in Buffer.** The stock solution of SBP-apo-AEQ hybrid protein was diluted to 1.0 x 10^{-7} M in 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer. Then, coelenterazine was added to a final concentration of 1 µg/mL and incubated at 4 °C
for 16 hours to form the active form of SBP-AEQ. A 1 M stock solution of sodium sulfate made in 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer was serially diluted in 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer. Twenty microliters of each sulfate concentration was added to a microtiter plate wells containing 80 µL of the hybrid protein. For the control samples, 20 µL of buffer was added to a microtiter plate wells containing 80 µL of hybrid protein. Replicates of four were performed for each concentration of sulfate and the control. After 15 minutes of incubation at room temperature, bioluminescence was triggered by injecting 100 µL of triggering buffer, and bioluminescence emission intensity was measured on the FLUOstar Optima luminometer (BMG Labtech, Cary, NC). The collected data was plotted as total intensity in relative light units (RLU) versus log [sulfate], and was fitted using the log (agonist) versus response non-linear fit of GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The percent change in signal was calculated by [(the average bioluminescence emission intensity of SBP-AEQ without sulfate (blank) - the average bioluminescence emission intensity of SBP-AEQ with 3.16 x 10^{-2} M sulfate) / the average bioluminescence emission intensity of SBP-AEQ without sulfate (blank)] times 100. The detection limits were determined by interpolating the average bioluminescence emission intensity of SBP-AEQ without sulfate plus three times the standard deviation of the blank.

**SBP-AEQ Response in Tap Water, Simulated Urine and Serum.** The same procedure outlined in the above section titled *SBP-AEQ Response in Buffer* is followed; however, all the sulfate standards and control solutions were prepared by using appropriate solutions. For the tap water a 1 M stock solution of sodium sulfate was prepared and serially diluted in tap water. For the simulated urine, a 1 M stock solution of sodium sulfate was prepared and serially diluted in simulated urine (Carolina Biological, Burlington, NC). Finally, for the serum, the sodium sulfate standards were prepared in 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer and diluted to ten fold into human serum from human male AB plasma (Sigma catalog # H4522). The serum controls consisted of 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer were diluted ten fold into serum. The detection limits were determined by interpolating
the bioluminescence emission intensity without sulfate plus three times the standard deviation of the blank to the correct concentration.

**Selectivity.** To determine the selectivity of the SBP-AEQ, the stock solution of SBP-apo-AEQ hybrid protein was diluted to $1.0 \times 10^{-7}$ M in 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer and coelenterazine was added to a final concentration of $1\mu$g/mL. One hundred millimolar solutions of sodium selenate, sodium nitrate, sodium arsenate, sodium perchlorate and sodium sulfate were prepared in 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer. Then, an aliquot of 20 µL of each analyte was added to a microtiter plate well containing 80 µL of the hybrid protein. Each analyte was measured in triplicates. After 15 minutes of incubation at room temperature, bioluminescence emission was triggered by injecting 100 µL of triggering buffer, and bioluminescence emission intensity was measured on the FLUOstar Optima luminometer (BMG Labtech, Cary, NC).

**Results and Discussion**

Molecular switches are selective detection tools that can potentially play a significant role in bioanalytical techniques. Herein, we report the design and preparation of a protein-based molecular switch for analytical sensing, specifically a protein-only switch. Protein-only switches for analytical sensing include switches with the receptor and signal transducer element within the same protein. The preparation of protein-only switches can be performed employing different strategies. In a classic example, Tsien et al. developed a hybrid protein by inserting calmodulin into split enhanced yellow fluorescent protein for the detection of calcium. In another example, Doi and Yanagawa site directly introduced TEM1 β-lactamase within the green fluorescent protein followed by random mutagenesis creating a drug screening method for TEM1 β-lactamase inhibitors. In our laboratory, we have previously demonstrated the creation of protein switches by rational design, which entailed preparing hybrid proteins comprised of the photoprotein aequorin and a binding protein capable of selective recognition of target ligands.
Herein we describe a new aequorin-based protein switch consisting of a hybrid protein of aequorin and the sulfate-binding protein (SBP-AEQ). To design a functional SBP-AEQ protein switch, the first challenge was splitting AEQ into two fragments that, upon interaction, could re-assemble into an active protein. AEQ has a highly ordered structure; therefore, the creation of a protein switch by the insertion of a second protein into the AEQ secondary structure must be done in a rational manner. Previously, we demonstrated that AEQ can function as a reporter molecule in a protein switch upon the insertion of another protein between residues 47 and 48 of the AEQ sequence. We followed this same strategy of rational design of the molecular switch by inserting the entire SBP sequence between residues 47 and 48 of aequorin with a six amino acid linker on each terminus of SBP so that, when sulfate ions bind to SBP, the conformational change known to occur in the hinge region of the protein is propagated to the split AEQ altering the intensity of light emitted.

To engineer the SBP-apo-AEQ hybrid protein, the gene sequence of wild-type SBP was amplified via PCR, and overlap PCR was used to amplify apo-AEQ with endonuclease restriction sites between two SGGGGS linkers that are inserted between residues 47 and 48, creating overlap-apo-AEQ. Overlap-apo-AEQ was ligated into pET-28a(+), which has a T7 promoter and His-tag followed by a multiple cloning site, so that the His-tag is on the N-terminus of the overlap-apo-AEQ creating pOLAEQ. The amplified SBP gene was then ligated into pOLAEQ generating pSBPAEQ. The pSBPAEQ plasmid was then transformed into chemically competent E. coli cells. The hybrid protein was overexpressed inside the E. coli cells upon induction with IPTG and the presence of the SBP-apo-AEQ protein was confirmed by running an SDS-PAGE where the presence of an intense band at 59 kDa (SBP - 35 kDa plus AEQ - 21.4 kDa, plus His-tag and linkers) was observed. The SBP-apo-AEQ protein was purified by immobilized metal affinity chromatography (IMAC) using Ni/NTA affinity resin under denaturing conditions with urea as the denaturing agent. The elution fractions contained the denatured SBP-apo-AEQ hybrid protein, as well as a contaminating band at approximately 49 kDa; consequently further purification was desirable. Therefore, the elution fractions were pooled together and dialyzed against 2 L of 30 mM Tris/HCl, pH
7.5, 2 mM EDTA, 4 M urea buffer to remove excess imidizole and adjust the pH for the subsequent ion exchange chromatography. Specifically, diethylaminoethyl (DEAE) sepharose resin was chosen as a weak anion exchanger. The bound proteins were eluted by running a buffer gradient containing no NaCl with 4 M urea to 1 M NaCl without urea. The SDS-PAGE of the purification demonstrated that the contaminating band at approximately 49 kDa was not retained by the anion exchange resin and was eluted in the flow through, while the SBP-apo-AEQ hybrid protein was successfully purified. The elution fractions containing the pure SBP-apo-AEQ protein were combined and the protein was renatured slowly via dialysis, which removes the urea from the denatured protein allowing the protein to refold into its normal three-dimensional structure.

To confirm that the hybrid apoprotein was refolded into its native form, it was incubated with the native coelenterazine chromophore, and then the molecular switch was characterized with regard to its bioluminescence emission and ability to respond to sulfate. The specific activity of the SBP-AEQ protein in the absence of sulfate was found to be $1.56 \times 10^9$ RLUs/mg and in the presence of sulfate was $1.97 \times 10^9$ RLUs/mg. On the other hand, the parent aequorin had a specific activity of $3.65 \times 10^{12}$ RLUs/mg. Despite the fact that the insertion of SBP into the AEQ structure lowered the activity of the photoprotein due to aequorin being in a less active conformation, it still allowed for the reunion of the two spliced fragments of AEQ, maintaining the emission of significant bioluminescence as well as the recognition ability of SBP for sulfate ions. Accordingly, the ligand bound to the hybrid protein leads to a more normal conformation of aequorin slightly increasing its specific activity. The wavelength of emission of SBP-AEQ with and without sulfate was found to be 470 nm, which is identical to that of the parent aequorin. In addition, the SBP-AEQ hybrid protein demonstrated the same type of flash kinetics as the unmodified aequorin. The half-life of the SBP-AEQ hybrid protein was found to be 1.62 s in the presence of sulfate, and 0.75 s in the absence of sulfate, which noticeably differs from the 0.60 s half-life of the parent aequorin. The longer half-life could be attributed to SBP inserted in-between the split halves of AEQ. As shown by the specific activity, the insertion of SBP reduces the luminescence activity to some extent; however, it does not considerably affect the spectral properties of aequorin. All these data
indicated that the bioluminescence emission mechanism has not been significantly altered, albeit it is less efficient than in the case of the unmodified aequorin.

To further understand the behavior of the molecular switch, we characterized the hybrid SBP-AEQ protein switch in terms of its tertiary structure. We observed that the specific activity increases (measured by bioluminescence emission) when sulfate is present, thus suggesting that the protein switch is functional. In order for the protein switch to be able to emit bioluminescence, the two fragments of the AEQ component of the hybrid protein must re-assemble in the presence of coelenterazine, and subsequently undergo a conformational change upon binding to Ca$^{2+}$. Given that the sulfate-binding protein is inserted in-between these two AEQ fragments, for re-assembly of the two AEQ fragments to occur they need to be brought together. This can only happen if the sulfate-binding protein, upon binding to the sulfate ions, undergoes a sufficient conformational change that will allow for the two AEQ fragments to come in close proximity. Thus, to further verify that the SBP-AEQ switch is functional, we examined whether the binding of sulfate to the hybrid protein results in an observable conformational change of the tertiary structure of the protein. To that end, near UV circular dichroism was employed demonstrating that when sulfate was added to SBP-AEQ the molar ellipticity changed (Figure 3.2). In general, a change in the molar ellipticity in the near UV region confirms that a change in the tertiary structure of the protein has occurred. This is indicated by the peak shift at 280 nm, as well as by the small peak shifts at 305 and 315 nm. Hence, all these data suggested that the SBP-AEQ hybrid protein molecular switch binds and senses sulfate in a similar manner and with a parallel mechanism as that of our previously developed AEQ-based protein switches.

Once the protein switch was characterized with regard to its mechanism of sensing and bioluminescence emission, several parameters were evaluated in order to optimize the response characteristics of the system. These parameters included the amount of hybrid protein to be used as a reagent, and then the incubation time needed for sulfate to bind effectively to the sulfate-binding protein, thus changing its conformation and, in turn, the emission of light by SBP-AEQ upon addition of Ca$^{2+}$. A study was
Figure 3.2. Near UV CD spectra. The spectra were collected with a JASCO J-810 spectrometer (JASCO Ltd., UK) with 1.0 nm bandwidth and using a 1.0 cm pathlength cuvette. The wavelength scans were corrected for the blank and converted into molar ellipticity. SBP-AEQ in the presence of sulfate (solid line) and SBP-AEQ in the absence of sulfate (---). The addition of sulfate to SBP-AEQ induced a change in the tertiary structure of the protein.
performed in order to determine the minimum amount of hybrid protein that results in the
emission of a bioluminescence signal above the background. For this, the stock SBP-apo-
AEQ was serially diluted in 30 mM Tris/HCl, pH7.5, 10 mM EDTA, 0.01% BSA buffer
and coelenterazine was added to each concentration of protein. Specifically, the hybrid
apoprotein was incubated with coelenterazine overnight at 4 °C, and after that the
bioluminescence emission was determined. The dilution study showed that the least
amount of SBP-AEQ protein that could be used was 1.0 x 10⁻⁷ M. Employing this
concentration of protein, a time study to determine the optimal response time of the
protein switch was performed. For this, SBP-AEQ was reacted with 3.16 x 10⁻³ M sulfate,
and the emission was determined at various time periods. Incubation times shorter than
15 min generated a negligible change in intensity, while incubation times of 15 min and
longer produced the maximum change in bioluminescence signal. Therefore, 15 min
reaction time was chosen as optimal in order to minimize the assay time.

The response of SBP-AEQ to sulfate ions was assessed by incubating varying
concentrations of sulfate ions for 15 min at RT with 1.0 x 10⁻⁷ M SBP-AEQ in the wells
of a microtiter plate. After this incubation time, the bioluminescence emission of the
protein switch was triggered by addition of LaCl₃, and the bioluminescence intensity was
measured. It should be mentioned that the bioluminescence of AEQ is generally triggered
by Ca²⁺ ions yet other ions, such as Sr²⁺, Cd²⁺, Y³⁺ and La³⁺, can also cause the emission
of significant bioluminescence. ²⁷ To avoid the formation of insoluble sulfate salts of the
metal ions employed to trigger bioluminescence, the solubilities of the salt products of
various triggering metals with sulfate ions were considered. Since calcium sulfate is
slightly insoluble in aqueous solutions and strontium sulfate is poorly water soluble and
insoluble in alkali solutions, neither calcium nor strontium ions were chosen as the
triggering agents for our sensing system that performs at pH 7.5. Cd²⁺ was not selected
because the quantum yield of the bioluminescence emission is reported to be lower than
that of the bioluminescent reactions triggered by the addition of Y³⁺ or La³⁺. ²⁷ LaCl₃ was
finally chosen as the triggering reagent given its simplicity of use and storage as
compared to metal oxides such as Y₂O₃. The calibration plot for sulfate (Figure 3.3)
Figure 3.3. Dose-response curve obtained with the protein switch when exposed to varying concentrations of sulfate in buffer. Specifically, sulfate solutions were incubated for 15 min with 1.0 x $10^{-7}$ M SBP-AEQ at RT. Bioluminescence was triggered with LaCl$_3$ on a FLUOstar Optima microtiter plate luminometer. Data points are an average of four replicates ± one standard deviation.
shows a dose-dependent relationship where an increase in sulfate concentration results in an increase in the bioluminescence emitted by the protein switch. From this doseresponse curve the detection limit for the sulfate ion is calculated to be $1.62 \times 10^{-4}$ M. The intra-assay reproducibility of the sensing system is represented by % relative standard deviation (RSD) values between 0.8% and 3.9%.

To evaluate the feasibility of using this SBP-AEQ sensing system in environmental and biological samples, calibration plots were generated in tap water, serum and simulated urine. For this, we employed the same assay format and conditions as the ones used in buffer. The dose-response curves for all three media were analogous to the curve obtained in buffer and virtually overlapped on top of each other, thus showing no significant matrix effect. The reproducibility of the sensing system in the samples tested was also similar to that in buffer. The analytical performance of the protein switch in the detection of sulfate in the three different samples is shown in Table 3.1. Interestingly, the working range in all three samples falls within the average sulfate levels in the respective matrices. The detection limit of the SBP-AEQ switch sensing system in tap water was $3.89 \times 10^{-4}$ M. According to the Environmental Protection Agency (EPA), sulfate in drinking water has a secondary maximum contaminant level, that is, the maximum concentration recommended to protect the organoleptic properties of drinking water, of $2.60 \times 10^{-3}$ M\textsuperscript{180}; therefore, this molecular switch could potentially be used to determine whether sulfate levels in water have exceeded the maximum concentration recommended. Furthermore, the dose-response curves obtained in spiked serum and simulated urine demonstrated the feasibility of using the protein switch for the detection of sulfate in clinical samples. The working range of the protein switch for the determination of sulfate in spiked serum falls within the average normal human serum sulfate concentration, which is $3.0 \times 10^{-4}$ M.\textsuperscript{176} Therefore, this protein switch sensing system could be an alternative rapid method of detecting sulfate in serum samples. Additionally, sulfate is excreted in urine in millimolar concentrations.\textsuperscript{169} The data in Table 3.1 shows that the linear range of the SBP-AEQ protein switch in simulated urine is in the millimolar range. A research study has shown that a group of healthy individuals
Table 3.1. Analytical performance characteristics of the SBP-AEQ protein switch.

Dose-response curves were plotted in GraphPad Prism 5.0 using log (agonist) versus response non-linear fit.

<table>
<thead>
<tr>
<th></th>
<th>Detection Limit (M)</th>
<th>Intra-assay RSD (%)</th>
<th>Working Range (M)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1.6 x 10⁻⁴</td>
<td>0.8 – 3.9</td>
<td>1.6 x 10⁻⁴ – 2.3 x 10⁻³</td>
<td>0.93</td>
</tr>
<tr>
<td>Tap water</td>
<td>3.9 x 10⁻¹</td>
<td>0.9 – 4.9</td>
<td>3.9 x 10⁻¹ – 4.7 x 10⁻³</td>
<td>0.91</td>
</tr>
<tr>
<td>Serum</td>
<td>1.9 x 10⁻⁴</td>
<td>0.7 – 4.6</td>
<td>1.9 x 10⁻⁴ – 2.3 x 10⁻³</td>
<td>0.93</td>
</tr>
<tr>
<td>Urine</td>
<td>1.9 x 10⁻⁴</td>
<td>1.0 – 4.3</td>
<td>1.9 x 10⁻⁴ – 3.5 x 10⁻³</td>
<td>0.91</td>
</tr>
</tbody>
</table>
had an average of $3.03 \times 10^{-3}$ M sulfate in their urine, whereas a group of patients with autism had an average of $6.82 \times 10^{-3}$ M sulfate excreted in their urine.\textsuperscript{169} The working range of the sensing system in simulated urine contains the average sulfate level of normal individuals showing the feasibility of employing SBP-AEQ for the detection of sulfate in the urine of both, healthy and autistic individuals. It should be noted that calcium ions, which are known to trigger aequorin bioluminescence, are present in biological and environmental samples; therefore, in order to prevent premature triggering of the bioluminescence emission of SBP-AEQ all the reactions should be performed in buffers containing EDTA as a calcium chelating agent.

Another analytical performance characteristic that needs to be studied is selectivity. It has been published that SBP binds with high selectivity to tetrahedral, fully ionized oxoanions.\textsuperscript{163} For this, SBP-AEQ was reacted with millimolar concentrations of various anions, and the response to these anions was evaluated in the same manner as for sulfate. It is noteworthy to mention that phosphate was not included due to the extremely low solubility of lanthanum phosphate. It was shown that nitrate, arsenate, chloride and perchlorate do not cause a significant response, which is reflected by a low increase in bioluminescence emission (see Figure 3.4). In contrast, it was found that the SBP-AEQ molecular switch responds to selenate ions, which could potentially interfere with the sensing of sulfate. However, reported levels of selenate in human serum and drinking water are much lower than the sulfate levels in these samples. Specifically, selenate concentration reported in drinking water is $3.85 \times 10^{-10}$ M\textsuperscript{181} and serum selenium concentration is $9.65 \times 10^{-7}$ M.\textsuperscript{182} Furthermore, similar to sulfate, selenate is a tetrahedral, fully ionized dianion, which is known to bind to native SBP with a 40 times lower affinity than sulfate.\textsuperscript{183} Taking into consideration the concentrations of selenate in water and serum, with respect to sulfate concentrations in the order of $10^{-3}-10^{-4}$ M, and the lower affinity of SBP to selenate, we speculate that it is unlikely for selenate to interfere with sulfate detection, especially for drinking water where the difference between normal selenate and sulfate concentration levels is approximately seven orders of magnitude. On the other hand, if human overexposure to selenate were to occur it is possible for selenate to interfere with sulfate detection in serum. Therefore, we foresee this sensing system
Figure 3.4. Selectivity studies employing the SBP-AEQ protein switch. Each anion was incubated for 15 min with $1.0 \times 10^{-7}$ M SBP-AEQ at RT. Bioluminescence was triggered with LaCl$_3$ on a FLUOstar Optima microtiter plate luminometer. Data points are an average of four replicates ± one standard deviation.
being employed as a rapid on-site screening system for sulfate followed by further analysis to confirm the actual levels of sulfate present, when values out of the normal range are obtained.

In conclusion, we have used genetic engineering to join two unrelated proteins, SBP and AEQ, for the development of a unique protein switch sensing system for sulfate ions. This protein switch provides an alternative to other methods of sulfate detection and is suited for on-site detection since it does not require any sample pretreatment and provides an answer in a relatively short period of time, i.e., within 15 min of the collection of the sample. The detection limit for this system was found to be $1.62 \times 10^{-4}$ M sulfate and the selectivity and reproducibility of the system were evaluated. Further, we demonstrated the possibility of using SBP-AEQ for the detection of sulfate in biological and environmental samples by analyzing sulfate in spiked drinking water, human serum and simulated urine.
CHAPTER FOUR

BIOLUMINESCENCE BINDING ASSAY FOR THE DETECTION OF HYDROXYLATED PCBS

Introduction

Polychlorinated biphenyls (PCBs) have long been investigated for their environmental and biological impact. PCBs are toxic and persistent chemicals that had been primarily used as insulating fluids and coolants in heavy-duty electrical equipment in power plants, industries and large buildings across the United States. Among the characteristics of PCBs that made them so desirable for these uses was their extreme stability. As a result of this stability, PCBs are highly resistant to degradation in nature, which leads to their accumulation in the environment as well as bioaccumulation in living organisms. PCBs are carcinogenic to humans, increasing the risks for certain cancers including melanoma, liver, biliary tract and intestinal cancers.\(^{156}\) In addition, they are toxic to the neuroendocrine, immune and reproductive systems.\(^{184}\) Even though the production, processing and utilization of PCBs were banned in the 1970s, PCBs are still a widely distributed and persistent environmental threat and, as a consequence, they continue to be studied extensively.

In bacteria and higher organisms, including humans, PCBs are biotransformed by cytochrome P-450 mono-oxygenases and metabolized to hydroxylated polychlorinated biphenyls (OH-PCBs) and methyl-sulfone PCBs.\(^ {185}\) The OH-PCBs are slightly more hydrophilic than PCBs, nevertheless, they are still slowly excreted due to selective binding to proteins and to retention in cells.\(^ {186}\) In fact, these compounds have been found in the blood of humans and wildlife.\(^ {187-195}\) OH-PCBs are emerging as dangerous persistent environmental contaminants; their toxicity may be greater than that of the parent PCBs, particularly with regard to endocrine disruption. A number of OH-PCBs have been identified as endocrine disruptors.\(^ {184,196-201}\) For example, 4-hydroxy-2,3,3’,4’,5-pentachlorobiphenyl was found to be weakly estrogenic, while 4-hydroxy-2,3,3’,4,5,5’,6-heptachlorobiphenyl and 4-hydroxy-2,2’,3,4’,5,5’-hexachlorobiphenyl
were found to be anti-estrogenic. The same study concluded that persistent OH-PCBs inhibit gap junction intercellular communication, which may lead to tumor promotion.\textsuperscript{184} Furthermore, 4-hydroxy-2,3,3',4,5,5',6-heptachlorobiphenyl exposure has been correlated with higher free thyroxine levels in newborns\textsuperscript{196} and 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl exposure with lower levels of thyroxine in fetal rat plasma and brain.\textsuperscript{197} Another study has shown that there is a significant association between maternal and umbilical cord 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl levels and slower mental development in children.\textsuperscript{202}

Hydroxylated polychlorinated biphenyls have also been detected in aquatic environments, possibly being produced through aerobic biodegradation\textsuperscript{203} or by reaction of the parent PCBs with hydroxyl radicals.\textsuperscript{204} These compounds have been quantified in rain, snow and surface water with higher levels recorded in snow and surface waters near sewage treatment plants,\textsuperscript{205} ground waters downstream of landfills\textsuperscript{206} and in near-shore surface waters.\textsuperscript{194} The persistent nature of OH-PCBs in the environment causes human and animal exposure, which, along with bioaccumulation in body tissues, can create negative health effects to those exposed. Thus, improved methods for the detection of OH-PCBs in the environment should help facilitate environmental clean-up efforts and limit their adverse health effects on humans.

Traditional detection methods for OH-PCBs have been mostly based on GC-MS and LC-MS analyses,\textsuperscript{207,208} which require a series of sample preparation steps, and are time consuming as well as costly. Recently, our group developed a whole cell biosensing system capable of detecting several OH-PCBs. For that, \textit{Escherichia coli} cells were transformed with a plasmid containing the gene for the bioluminescent reporter protein, bacterial luciferase, under the control of the regulatory protein, HbpR, which binds OH-PCBs. When OH-PCBs enter the cell and bind to HbpR, gene transcription is activated, the luciferase is expressed in a dose-dependent fashion and bioluminescence is measured. A detection limit of $1.0 \times 10^{-8}$ M OH-PCBs in environmental and serum samples was achieved. This whole cell sensing system represents a more rapid and cost-effective approach to OH-PCB detection and is amenable to incorporation into portable field
While this sensing system demonstrated advantages over traditional methods, employing a protein instead of an entire cell as the sensing element has the advantage of further reducing the response times since the diffusion of the analyte into the cell as well as activation of the cell machinery to produce the reporter molecule are eliminated. Additionally, it is easier to incorporate proteins into miniaturized devices for field analyses than it is to incorporate and maintain living cells. With these improvements in mind, we designed and developed a protein based bioluminescence inhibition assay system that could lead to a more rapid method of detection of OH-PCBs, especially for onsite screening of environmental and biological samples.

The design of this protein based inhibition assay utilizes the bioluminescent protein aequorin (AEQ) as both the recognition and reporter molecule. AEQ is a photoprotein found in the jellyfish *Aequorea victoria*, which bioluminates in the presence of calcium ions, coelenterazine and molecular oxygen. Aequorin consists of an apoprotein, apoaequorin (apoAEQ), composed of 189 amino acids that form a hydrophobic pocket, where an organic prosthetic group called coelenterazine is non-covalently bound. In addition, AEQ has four EF hand structures, three of which bind calcium, and three triads of amino acids each consisting of tryptophan, tyrosine and histidine residues within its hydrophobic pocket that stabilize coelenterazine for bioluminescence (Figure 4.1). When calcium is added to AEQ, the protein undergoes a conformational change that leads to oxidation of coelenterazine to coelenteramide and release of CO₂ and light at 469 nm. We have taken advantage of this phenomenon, and studied the interaction between the photoprotein and different organic molecules to assess the binding selectivity of the amino acids within the hydrophobic pocket. We hypothesized that the three-dimensional structure resulting from the amino acids folding in space results in differential binding of organic molecules, and perhaps displacement or blockage of the interaction between the coelenterazine and the apoprotein, which in turn, alters the emission of bioluminescence. Our studies focused on investigating various molecules that potentially can fit within the pocket. Comparison of coelenterazine and the OH-PCBs showed that there are similarities in their structures and suggested the potential for the binding of OH-PCBs to the hydrophobic pocket of aequorin. Binding of OH-PCBs
Figure 4.1. The 3-D crystal structure of AEQ with coelenterazine in the hydrophobic pocket. Protein data bank structure 1EJ3.
to the hydrophobic pocket could displace coelenterazine or alter its binding to aequorin, resulting in a change in bioluminescent activity. Hence, we decided to examine the effect of the OH-PCBs on aequorin activity with the goal of exploring the potential use of aequorin in a bioluminescent inhibition assay for these compounds. Interestingly, we demonstrated that various OH-PCBs can bind to AEQ, causing a dose-dependent decrease in the bioluminescence of AEQ. We took advantage of this concentration-dependent modulation of the bioluminescence signal by the OH-PCBs to develop an inhibition assay for this family of toxic compounds.

We achieved a one-phase screening assay that requires no separation steps or sample preparation, thus, it is rapid, inexpensive, simple and amenable to miniaturization and on-site testing. A unique feature of the developed inhibition assay is that AEQ plays a dual role, namely it is the recognition as well as the signal generating molecule, thus not requiring labeling or gene fusion methods to couple the recognition and reporting elements. Herein, dose-response curves for various OH-PCBs were also constructed in serum and tap water to prove the feasibility of employing our method in the detection of this family of compounds in biomedical as well as in environmental samples. In addition, we have characterized AEQ in the presence of several OH-PCBs and performed mechanistic studies to understand how these OH-PCBs interact with AEQ.

**Materials and Methods**

**Materials.** Tris free base – Tris (hydroxymethyl) amino methane was purchased from Serva (Heidelberg, Germany). Glycine, sodium chloride, disodium ethylenediaminetetraacetate (EDTA), Luria-Bertani (LB) Agar, and LB Broth were purchased from Fischer Scientific (Fair Lawn, NJ). Albumin from bovine serum (BSA), calcium chloride, 2-hydroxybiphenyl, sodium phosphate monohydrate, kanamycin monosulfate, ethidium bromide, dimethyl sulfoxide (DMSO) anhydrous, nicotine hydrogen tartrate salt, amantadine hydrochloride, 2,2’-dibromobiphenyl, 3-chlorobenzoic acid, bisphenyl-A, 4-chlorocatechol, 4-chlorophenyl and pentachlorophenol were purchased from Sigma-Aldrich (St. Louis, MO). Sodium Dodecyl Sulfate (SDS) was purchased from Curtin Mathesin Scientific. (Houston, TX). Coelenterazine was
purchased from Gold Biotechnology. (St. Louis, MO). TAPP (3-trifluoromethylphenothiazine-10-yl) propyamine was purchased from Quanta BioDesign (Powel, OH). The Bradford protein assay kit was purchased from Bio-Rad Laboratories. (Hercules, CA). Mini-prep kits and gel extraction kits were purchased from Qiagen (Valencia, CA). The plasmid pET28(a)+ and BL21(DE3) competent cells were purchased from Novagen (Madison, WI). Gel code blue stain and 3,500 MWCO Slide-A-Lyzer dialysis cassettes were purchased from Pierce (Rockford, IL). UltraAmicon 10,000 MWCO centrifuge devices were purchased from Millipore (Cork, Ireland). Butyl Sepharose 4 fast flow and DEAE Sepharose Fast Flow were purchased from Amersham Bioscience (Uppsala, Sweden). OH-PCBs and PCBs were purchased from Accustandard. (New Haven, CT). All chemicals were reagent grade or better and solutions were prepared using deionized reverse osmosis (Milli-Q Water Purification System, Millipore, Bedford, MA) water.

**Construction of Plasmid pIN4-AEQ.** The apoaequorin cysteine-free mutant gene was used as the template for the PCR. Primers 1 and 2 were used to amplify the aequorin gene where primer 1 introduced an *Eco*RI restriction site (underlined) on the 5’ end of the coding sequence and primer 2 introduced an *Hind*III site (underlined) on the 3’ end.

1. GTGGAATTCCATGGTGAAACTGACCAGCGACTTCGACAACCCAAGATG

2. CACAGGGCTTTTAGGGGACAGCTCCACCCTAGAGCTTTTCGGAAGCAGG

ATC

The purified PCR product was subcloned in the pCR2.1-TOPO vector, using the TOPO TA Cloning method by Invitrogen (Carlsbad, CA). The cloned plasmid DNA was isolated using the Qiagen Mini Prep Kit (Valencia, CA). Both the AEQ DNA and the plasmid, pIN4, were digested with *Eco*RI and *Hind* III restriction enzymes. Then, the digested aequorin DNA insert was ligated into pIN4 yielding pIN4-AEQ. The pIN4-AEQ vector was transformed into *E. coli* TOP10 cells, and the DNA was isolated for sequencing. The constructed DNA was sequenced at the University of Kentucky.
Advanced Genetics Technology Center (AGTC) to confirm the presence of the in frame AEQ gene in the plasmid.

**Expression and Purification of AEQ.** The bacterial cells were grown overnight at 37 °C, 250 rpm in 5 mL of LB broth containing 100 μg/mL ampicillin. This culture was used to inoculate 500 mL of LB broth containing 100 μg/mL ampicillin, and this was grown overnight at 37 °C, 250 rpm. The cells were harvested by centrifugation at 22,100 x g at 4 °C for 15 minutes. The medium (supernatant) was saved and the expressed aequorin in the medium was precipitated by adding glacial acetic acid until the pH reached approximately 4.0. The precipitated proteins were isolated by centrifugation at 22,100 x g at 4 °C for 30 minutes. The pellet was resuspended in 30 mM Tris/HCl, pH 7.5 buffer containing 2 mM EDTA (Buffer A) and the pH was adjusted to 7.5 with 5 M sodium hydroxide. This resuspended protein was gently shaken at 4 °C for 30 minutes. The suspension was centrifuged at 22,100 x g at 4 °C for 15 min to remove any particulates and obtain the supernatant containing the protein in solution. The protein was purified using a BioCAD Sprint Perfusion chromatography system from PerSeptive Biosystems (Framingham, MA) with a DEAE Sepharose Fast Flow-ion exchange column (20 mm diameter x 70 mm). The column was equilibrated with Buffer A. The supernatant containing the protein was loaded onto the column, and the column was washed with three column volumes of the equilibration buffer. The protein was eluted using a gradient of 0 % (no elution buffer) to 50 % elution buffer (Buffer A containing 1 M NaCl) over ten column volumes. Five-milliliter fractions were collected. An SDS-PAGE was run to verify the fractions containing the apoprotein, which were then pooled together. The combined fractions were further purified using the BioCAD Sprint Perfusion chromatography system with a Butyl Sepharose hydrophobic interaction chromatography column (20 mm diameter x 70 mm). To do this, ammonium sulfate was added to the combined DEAE fractions until the ammonium sulfate concentration was 1.0 M. The column was equilibrated with 20 mM Bis-Tris, pH 7.2 buffer containing 2 mM EDTA and 1 M ammonium sulfate. The fractions containing the protein with ammonium sulfate were loaded on the column and the column was washed with three column volumes of the equilibration buffer. The column was further washed with 20% equilibration buffer,
80% elution buffer (20 mM Bis-Tris, pH 7.2, 2 mM EDTA), and then the apoaequorin was eluted using a gradient of 80% to 100% elution buffer over two column volumes. Five-milliliter fractions were collected. The purity of the protein was confirmed by using SDS-PAGE. The fractions containing the pure apoprotein were pooled together and dialyzed for 16 hours at 4 °C against 2 L of Buffer A using 3500 MWCO Pierce dialysis cassettes. The protein concentration was determined by the Bradford protein assay, with BSA as a standard. Protein was stored at -80 °C until use.

**Dilution Study for AEQ.** The apoprotein stock solution was serially diluted in Buffer A containing 20% DMSO. In a polystyrene microtiter plate, 100 µL of each concentration of diluted protein, in triplicates, was incubated with a three times molar excess of native coelenterazine overnight at 4 °C. Bioluminescence was triggered by injecting 100 µL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl₂ buffer and light intensity was measured using a FLUOstar Optima luminometer (BMG Labtech, Cary, NC).

**Effect of DMSO and OH-PCB on AEQ.** The stock solution of apoaequorin protein (1.21 × 10⁻⁵ M) was diluted to 1.21 × 10⁻⁷ M in Buffer A, and incubated with a three times molar excess of coelenterazine at 4 °C for 18 h. To evaluate the DMSO effect, four samples were prepared: AEQ with a) 10% DMSO, b) 20% DMSO, c) 10% Buffer A and d) 20% Buffer A. For a), an aliquot of 180 µL of protein was mixed with 20 µL of DMSO and for b), an aliquot of 160 µL of protein was mixed with 40 µL of DMSO. The same dilutions were performed for c) and d), using Buffer A instead of DMSO. After mixing the protein with DMSO or buffer, 10 µL of solution was added to a disposable glass tube. Bioluminescence was triggered by injecting 50 µL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl₂ buffer, and bioluminescence was measured on the Optocomp I luminometer (MGM Instruments, Hamden, CT). The same procedure was repeated for the model OH-PCB, 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl, by adding the model OH-PCB dissolved in DMSO in place of the DMSO alone or buffer.

**Coelenterazine Dose-Response Curve - EC₅₀ Determination.** The stock solution of apoaequorin (1.21 × 10⁻⁵ M) was diluted to 1.21 × 10⁻⁷ M in Buffer A. Aliquots of 80 µL
of diluted protein were added to the wells of a microtiter plate containing 20 µL of DMSO. Various concentrations of native coelenterazine were then added, in triplicates (final concentrations ranging from 1.0 x 10^{-5} M to 1.0 x 10^{-11} M). The samples were mixed and stored at 4 °C for 18 h. Bioluminescence was triggered by injecting 100 µL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl₂ buffer, and bioluminescence was measured on the FLUOstar Optima luminometer (BMG Labtech, Cary, NC). The bioluminescence signal was collected for 6-s and the EC₅₀ value was calculated using non-linear regression fit in GraphPad Prism 5.0 (Sigmoidal Dose-Response (variable slope)).

**Association Study – Determining the optimum concentration of coelenterazine.** The stock solution of apoaequorin (1.21 x 10^{-5} M) was diluted to 1.21 x 10^{-7} M in Buffer A. A 1.0 x 10^{-2} M stock solution of the 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl in DMSO was serially diluted in DMSO. Three assays were set up with different final concentrations of coelenterazine: 1.0 x 10^{-6} M, 6.1 x 10^{-8} M and 5.0 x 10^{-10} M. For each assay, an aliquot of 20 µL of each concentration of 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl was added to 80 µL diluted apoAEQ in triplicates in polystyrene microtiter plate wells. Also, a blank sample containing 20 µL DMSO was prepared in triplicates. For each assay, the appropriate final concentration of coelenterazine was added, mixed and stored at 4 °C for 18 h. Bioluminescence was triggered by injecting 100 µL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl₂ buffer, and bioluminescence was measured on the FLUOstar Optima luminometer (BMG Labtech, Cary, NC). The bioluminescence signal was collected for 6-s and the EC₅₀ values were calculated using non-linear regression fit in GraphPad Prism 5.0 (One-site – fit logIC₅₀).

**Time Study.** The stock solution of apoaequorin (1.21 x 10^{-5} M) was diluted to 1.21 x 10^{-7} M in Buffer A. A 1.0 x 10^{-2} M stock solution of the 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl in DMSO was serially diluted in DMSO. An aliquot of 20 µL of each concentration of 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl was added to 80 µL diluted apoAEQ in triplicates, for each time point, in polystyrene microtiter plate wells. Also, a blank sample containing 20 µL DMSO was prepared in triplicates, for each time point. A final concentration of 5.0 x 10^{-10} M coelenterazine was added to each well,
mixed and stored at 4 °C for 2, 4 and 18 h. At each time point, bioluminescence was triggered by injecting 100 μL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl₂ buffer, and bioluminescence was measured on the FLUOstar Optima luminometer (BMG Labtech, Cary, NC). The bioluminescence signal was collected for 6-s and the IC₅₀ values were calculated using non-linear regression fit in GraphPad Prism 5.0 (One-site – fit logIC₅₀).

**Dose-Response Curves.** The protocol was the same for all OH-PCBs studied. The stock solution of apoaequorin (1.21 x 10⁻⁵ M) was diluted to 1.21 x 10⁻⁷ M in Buffer A. A 1.0 x 10⁻² M stock of each OH-PCB in DMSO was serially diluted in DMSO. An aliquot of 20 μL of each concentration of OH-PCB was added to 80 μL of the diluted apoAEQ in triplicates in polystyrene microtiter plate wells. Also, a blank sample containing 20 μL DMSO was prepared in triplicates. A final concentration of 5.0 x 10⁻¹⁰ M coelenterazine was added to each well, mixed and stored at 4 °C for 2 h. Bioluminescence was triggered by injecting 100 μL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl₂ buffer, and bioluminescence was measured on the FLUOstar Optima luminometer (BMG Labtech, Cary, NC). The bioluminescence signal was collected for 6-s and the IC₅₀ values were calculated using non-linear regression fit in GraphPad Prism 5.0 (One-site – fit logIC₅₀).

**Circular Dichroism (CD) Studies.** Apoaequorin was concentrated and buffer exchanged in a low salt buffer (5 mM phosphate buffer, pH 7.0 containing 2 mM EDTA) before the CD measurement. This was achieved using Amicon Ultra 3500 MWCO centrifugal filter devices (Millipore Corp.) according to the manufacturer’s specifications. For sample 1 (apoaequorin without OH-PCB) apoaequorin was incubated for 15 minutes at room temperature with an aliquot of DMSO corresponding to 20% of the total volume. For sample 2 (apoaequorin with OH-PCB) apoaequorin was incubated for 15 minutes at room temperature with an aliquot of DMSO corresponding to 20% of the total volume, containing 5.0 x 10⁻⁴ M 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl. Aequorin was prepared by incubating apoaequorin overnight at 4 °C with 3 times molar excess of coelenterazine. For sample 3 (aequorin without OH-PCB), aequorin was incubated for 15 minutes at room temperature with an aliquot of DMSO corresponding to 20% of the total volume. For sample 4 (aequorin with OH-PCB), aequorin was incubated for 15 minutes
at room temperature with an aliquot of DMSO corresponding to 20% of the total volume, containing \(5.0 \times 10^{-4}\) M 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl. Blanks were prepared for all four samples. For sample 1 the blank was composed of the low salt buffer with an aliquot of DMSO corresponding to 20% of the total volume, and for sample 2 the blank was composed of the low salt buffer with an aliquot of DMSO corresponding to 20% of the total volume, containing \(5.0 \times 10^{-4}\) M 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl. For sample 3 the blank was composed of the low salt buffer with an aliquot of DMSO corresponding to 20% of the total volume and the appropriate amount of coelenterazine, and for sample 4 the blank was composed of the low salt buffer with an aliquot of DMSO corresponding to 20% of the total volume, containing 5.0 \(\times\) 10\(^{-4}\) M 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl, and the appropriate amount of coelenterazine. For all four samples and blanks, the CD spectra were collected with a JASCO J-810 spectrometer (JASCO Ltd., UK) with a 1 nm bandwidth using a 1.0 cm pathlength cuvette for the near UV scans. The wavelength scan was performed at 20 °C, and corrected for the blank. The concentration of protein in each sample was determined by the Bradford assay, and the spectra were normalized to the protein concentration.

**Determination of apparent \(K_{d}\)** Determination of the apparent dissociation constants for apoaequorin and coelenterazine as well as apoaequorin and 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl was performed according to Eremeeva et. al.\(^2\) Apoaequorin was titrated with native coelenterazine or 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl. Briefly, the stock solution of apoaequorin (1.21 \(\times\) 10\(^{-5}\) M) was diluted to 1.22 \(\times\) 10\(^{-6}\) M in Buffer A. Then, 2.36 \(\times\) 10\(^{-4}\) M stock solutions of coelenterazine and of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl were made in methanol and in DMSO, respectively. Six titrations were performed: apoaequorin was titrated with coelenterazine (S1), apoaequorin with methanol as control (C1), buffer with coelenterazine as blank (B1), apoaequorin with 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl (S2), apoaequorin with DMSO as control (C2) and buffer with 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl as blank (B2). For each titration, 180 \(\mu\)L of protein or buffer was added to a 1-cm quartz cuvette and the ligand or solvent was titrated stepwise. After each addition of titrant, the sample was mixed and fluorescence was measured using an excitation wavelength of 295
nm and emission wavelength of 324 nm. Measurements were performed with a Photon Technology International Quanta master 40 steady state spectrofluorometer with 4 nm slits. All titrations were performed in triplicates. The $K_d$ was calculated using binding-saturation fit in GraphPad Prism 5.0 (One Site-Specific Binding).

**Bioluminescence Emission Spectra.** The procedure was the same for all OH-PCBs studied. The stock solution of apoaequorin (1.21 x 10^{-5} M) was diluted to 1.21 x 10^{-7} M in Buffer A. An aliquot of the 1.0 x 10^{-2} M stock solution of each OH-PCB in DMSO was serially diluted in DMSO. The final concentrations of these OH-PCB solutions corresponded to the IC$_{50}$ values (determined from the dose-response curves) of each OH-PCB (Table 1). Then, these OH-PCB solutions were mixed with the diluted apoAEQ in triplicates in a polystyrene microtiter plate. The blank samples containing an aliquot of DMSO without OH-PCB, corresponding to 20% of the total volume, were prepared in triplicates. For each sample, a final concentration of 5.0 x 10^{-10} M native coelenterazine was added, mixed and stored at 4 °C for 2 h. Bioluminescence was triggered by injection of 100 μL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl$_2$ buffer. The bioluminescence signal was collected at wavelengths between 400 and 700 nm on a Spectro System Spectrographic Luminometer custom built by Sciencewares, Inc. (Falmouth, MA).

**Bioluminescence Half-Life Study.** The procedure was the same for all OH-PCBs studied. The stock solution of apoaequorin (1.21 x 10^{-5} M) was diluted to 1.21 x 10^{-7} M in Buffer A. An aliquot of the 1.0 x 10^{-2} M stock solution of each OH-PCB in DMSO was serially diluted using DMSO as the diluent. The final concentrations of these OH-PCB solutions corresponded to the IC$_{50}$ values (determined from the dose-response curves) of each OH-PCB (Table 1). Then, these OH-PCB solutions were mixed with the diluted apoAEQ in triplicates in a polystyrene microtiter plate. The blank samples containing an aliquot of DMSO without OH-PCB, corresponding to 20% of the total volume, were prepared in triplicates. For each sample, a final concentration of 5.0 x 10^{-10} M native coelenterazine was added, mixed and stored at 4 °C for 2 h. Bioluminescence was triggered by injecting 100 μL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl$_2$ buffer, and then measured on the FLUOstar Optima luminometer (BMG Labtech, Cary, NC). The
The bioluminescence signal was collected for 25-s and the half-lives were calculated using the one phase exponential decay kinetics equation in GraphPad Prism 5.0.

**Gaddum/Schild Response.** The stock solution of apoaequorin (1.21 x 10^-5 M) was diluted to 1.21 x 10^-7 M in Buffer A. A 1.0 x 10^-2 M stock of the 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl made in DMSO was serially diluted using DMSO as the diluent. Four different assays were set up with different final concentrations of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl: 0, 1.0 x 10^-6 M, 1.0 x 10^-7 M and 1.0 x 10^-8 M. For this, an aliquot, corresponding to 20% of the total assay volume, of each concentration of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl was added to the diluted apoAEQ in triplicates in polystyrene microtiter plate wells. Also, a blank sample containing an aliquot of DMSO without OH-PCB, corresponding to 20% of the total assay volume, was prepared in triplicates. For each sample, the appropriate final concentration of coelenterazine was added, mixed and stored at 4 °C for 18 h. Bioluminescence was triggered by injecting 100 μL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl2 buffer, and then measured on the FLUOstar Optima luminometer (BMG Labtech, Cary, NC). The bioluminescence signal was collected for 6-s and the Schild slope was calculated using the Gaddum/Schild EC50 shift parameters in GraphPad Prism 5.0.

**Comparison of Kd and Bmax.** The stock solution of apoaequorin (1.21 x 10^-5 M) was diluted to 1.22 x10^-6 M in Buffer A. Apoaequorin with or without 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl was titrated with native coelenterazine. For the sample containing the OH-PCB, 1.22 μM apoAEQ was incubated with 1.22 μM 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl and for the sample without OH-PCB, 1.22 μM apoAEQ was incubated with the appropriate amount of DMSO. Incubations were performed overnight at 4 °C. The 2.36 x 10^-4 M stock solution of coelenterazine was made in methanol. Then, six titrations were set up: apoaequorin containing DMSO was titrated with coelenterazine (S1), apoaequorin/DMSO with methanol (C1), buffer/DMSO with coelenterazine (B1), apoaequorin with 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl with coelenterazine (S2), apoaequorin/2OH2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl with methanol (C2) and buffer/2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl with
coelenterazine (B2). For each titration, an aliquot of 180 µL of protein solution or buffer was added to a 1-cm quartz cuvette and coelenterazine or methanol was titrated stepwise. After each addition of titrant, the sample was mixed and fluorescence was measured using a Photon Technology International master 40 steady state spectrofluorometer with 4 nm slits. All titrations were performed in triplicates. The $K_d$ and $B_{\text{max}}$ were calculated using binding-saturation fit in GraphPad Prism 5.0 (One Site-Specific Binding).

**Structural Characteristics.** The dihedral angles between the two rings of the OH-PCBs were calculated in Chem3D Pro12.0 using MM2 force field parameters during energy minimization. The molecular surface volumes were calculated using Connolly solvent excluded volume parameters after the energy minimization was performed. ClogP values were calculated in ChemDraw Ultra 12.0 using BioByte’s Bio-loom software.

**Results and Discussion**

In 1961, Shimomura et al. isolated aequorin from the jellyfish *Aequorea victoria*, and since then, AEQ has been characterized and extensively studied. AEQ is a 189 amino acid, calcium-binding photoprotein, consisting of the apoprotein, apoaequorin, and the prosthetic group, coelenterazine. When Ca$^{2+}$ binds to aequorin, the protein undergoes a conformational change and, through oxidation, the coelenterazine is converted into an excited state of coelenteramide and CO$_2$. As the excited coelenteramide relaxes to the ground state, blue light (wavelength = ~470 nm) is emitted. Employing AEQ as a reporting element has several advantages. In contrast to fluorescence, bioluminescence does not suffer from background emission as there is no need for the use of an excitation source for the photoprotein to emit light. When employing AEQ, background interference is avoided, and thus AEQ-based assays often render very sensitive detection. Furthermore, our group and that of others have demonstrated that AEQ can be detected at attomole levels. Due to the remarkably low limits of detection achieved when using aequorin, this photoprotein has been employed in many bioanalytical applications, including, as a calcium indicator in living cells and for *in vivo* imaging, as a reporter in DNA hybridization assays and immunoassays, and as the signal generating molecule in molecular switch sensing systems.
Herein, we have developed a sensitive, AEQ-based bioluminescence inhibition assay for the detection of OH-PCBs. A unique feature of this inhibition assay is that the AEQ plays a dual role, namely it is the recognition as well as the signal generating molecule. Specifically, upon incubation of apoAEQ with various OH-PCBs and coelenterazine, the amount of bioluminescence decreases as the concentration of the OH-PCB increases, making it possible to determine the concentration of OH-PCB in a sample.

The wild-type AEQ contains three cysteine residues; however, Kurose et al. prepared a cysteine-free mutant of AEQ and demonstrated that this cysteine-free variant had bioluminescent activity that was 10 to 20% greater than that of the wild-type photoprotein. In addition, the wild-type aequorin requires the presence of a reducing agent for the efficient binding of coelenterazine, while the cysteine-free aequorin does not. Given these characteristics of the cysteine-free AEQ that simplify its use and potentially increase the sensitivity of an assay employing it, we chose to use the cysteine-free aequorin in our studies. The E. coli system utilized for the expression of AEQ results in the release of the protein into the culture medium allowing for a simplified purification procedure. The protein was purified to >95% purity as determined by SDS-PAGE. Characterization of the purified protein showed that AEQ had a specific activity of $4.4 \times 10^{12}$ RLU/mg, a 0.59 s half-life and a 470 nm emission maximum.

The purified active AEQ was used to develop our inhibition assay for OH-PCBs. Initial experiments were performed by incubation of eight selected OH-PCBs, which ranged from one to five chlorine substituents with one or two hydroxyl groups, with apoaequorin and coelenterazine demonstrating that all these OH-PCBs caused a significant decrease in bioluminescence intensity. Since the OH-PCBs must be dissolved in DMSO, the effect of DMSO on AEQ activity was first examined. It was found that DMSO did not decrease AEQ bioluminescence activity; in fact the activity of AEQ was increased in the presence of DMSO by approximately 18%. However, the increase in activity did not affect our assay given that our experiments showed that various OH-PCBs decreased the bioluminescence of AEQ. Out of the eight compounds studied, 2'-hydroxy-2',3',4',5,5'-pentachlorobiphenyl had the most significant effect, a 99%
decrease of AEQ bioluminescence activity; therefore, it was chosen as a model compound to develop and optimize our system.

In a typical inhibition assay format, the binding of a single concentration of labeled ligand is measured in the presence of various concentrations of unlabeled ligand. Here, coelenterazine takes the place of the labeled ligand, since it is needed to produce the signal and the OH-PCB would be considered the unlabeled ligand, since it may compete with the coelenterazine for binding to aequorin. For the development of this assay, three parameters, namely the concentration of apoaequorin, the concentration of coelenterazine and the incubation time were evaluated for optimized analytical performance. The apoaequorin concentration chosen was \(1.23 \times 10^{-7}\) M (\(9.68 \times 10^{-8}\) M final) so that the number of protein molecules was low enough for competition to occur and there was sufficient activity for the decrease in bioluminescence to be observed in the presence of the OH-PCB. An association study was performed in order to determine the concentration of coelenterazine to be utilized in our inhibition assay. In standard competitive inhibition binding experiments, higher concentrations of labeled ligand (coelenterazine) require larger concentrations of unlabeled ligand (OH-PCB) to compete for half of the receptor binding sites. Assuming our assay performs in a competitive inhibition manner we optimized the assay to employ the least amount of coelenterazine required to get a good bioluminescent signal, so that lower concentrations of OH-PCB would be needed for competition and inhibition of bioluminescence, thus yielding better detection limits. For this, \(1.21 \times 10^{-7}\) M apoAEQ in the presence of an amount of DMSO corresponding to 20\% of the total volume was incubated with various concentrations of coelenterazine to produce AEQ (Figure 4.2). From this data, the EC\(_{50}\) (concentration of ligand (coelenterazine) that corresponds to 50\% of the maximum response elicited by the aforementioned ligand) was determined to be \(6.18 \times 10^{-8}\) M of coelenterazine. Determining the EC\(_{50}\) value gave us a basis for the concentrations of coelenterazine to evaluate in the assay development. Thus, coelenterazine concentrations at, above and below the EC\(_{50}\) value were studied in the presence of varying concentrations of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl. Figure 4.3 shows that all three coelenterazine concentrations yield dose-response curves with the IC\(_{50}\) values for the OH-PCB
Figure 4.2. Response curve for coelenterazine obtained by incubating apoaequorin (1.23 x 10^{-7} M) with varying concentrations of native coelenterazine. The data points are an average of three measurements ± 1 standard deviation. Plotted in GraphPad Prism 5.0 (Sigmoidal Dose-Response (variable slope)).
Figure 4.3. Dose-response curves for 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl obtained by incubating a known concentration of apoAEQ (1.23 x 10^{-7} M) with varying concentrations of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl in the presence of a given concentration of coelenterazine for each of the curves shown. The red curve corresponds to a concentration of 1.0 x 10^{-6} M coelenterazine, the blue to 6.1 x 10^{-8} M coelenterazine, the black to 5.0 x 10^{-10} M coelenterazine. The data points are an average of three measurements ± 1 standard deviation. Plotted in GraphPad Prism 5.0 using One Site Fit IC_{50} non-linear fit.
decreasing as the coelenterazine concentration decreases. The IC₅₀ is the half maximal inhibitory concentration, which is a measure of the effectiveness of a ligand in inhibiting a function, in this case bioluminescence, thus demonstrating the functional strength of the inhibitor. A concentration of 5.0 x 10⁻¹⁰ M coelenterazine was chosen as the optimal concentration given that it produced the lowest detection limit of 3.3 x 10⁻⁸ M of 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl.

Using the concentrations of apoAEQ and coelenterazine determined to be optimal in the above experiments, a time study was performed to determine the optimum incubation time required to produce the best response in the presence of varying concentrations of 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl. The data in Figure 4.4 demonstrates that after two, four and sixteen hours of incubation with coelenterazine, the responses to 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl are similar. Incubation periods of less than two hours did not yield significant bioluminescence. In order to reduce the overall assay time we selected an incubation time of two hours given that the detection limits are similar for all incubation periods tested.

Using 1.23 x 10⁻⁷ M apoaequorin, 5.0 x 10⁻¹⁰ M coelenterazine and two hours of incubation, which were found to be optimal for the assay, other OH-PCBs were evaluated and dose-response curves obtained (Table 4.1). The results show that the different OH-PCBs yield a variety of IC₅₀ values and detection limits. The lowest detection limits were obtained with 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl and 2-hydroxy-2’,3,4’,5,6–pentachlorobiphenyl. Conversely, 3,4-dihydroxy-3’4’-dichlorobiphenyl and 3-hydroxy-4’-chlorobiphenyl did not produce a decrease in the bioluminescence emission of AEQ. Comparisons of the structures of the OH-PCBs and their IC₅₀ values can lead us to speculate about what structural features of the OH-PCBs are important for the inhibition of aequorin activity. The effectiveness of this inhibition appears to be related to the hydrophobicity, the dihedral angle between the two rings of the biphenyl and the molecular surface volume of the OH-PCB. To gain some insight into how these parameters might affect the response of aequorin to the OH-PCBs, the ClogP values, dihedral angles and molecular surface volumes of each OH-PCB were estimated using
Figure 4.4. Time Study. The curves shown were obtained by incubating fixed concentrations of apoAEQ (1.21 x 10^{-7} M) and coelenterazine (5.0 x 10^{-10} M) with varying concentrations of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl for different times: 2 h – blue, 4 h – red, 16 h – black. The data points are an average of three measurements ± 1 standard deviation. Plotted in GraphPad Prism 5.0 using One Site Fit IC_{50} non-linear fit.
Table 4.1. Characteristics of the OH-PCBs evaluated in the AEQ bioluminescence inhibition assay. The coplanar OH-PCBs are in red and the non-coplanar OH-PCBs are in black. The detection limit was defined as the average signal of the blank (reaction mixture without OH-PCB) minus 3 standard deviations. The x’s stand for no response. The emission maximum ($\lambda$ max) and half-life were determined in the presence of the IC$_{50}$ concentration of the OH-PCB. The emission maximum and half-life in the absence of OH-PCB were found to be 472 nm and 0.86 s, respectively. The ClogP was calculated in ChemDraw Ultra. The dihedral angle and molecular surface volume were calculated in Chem3D Pro.

<table>
<thead>
<tr>
<th>OH-PCB</th>
<th>Detection Limit (µM)</th>
<th>IC$_{50}$(µM)</th>
<th>$\lambda$ max (nm)</th>
<th>Half-life (s)</th>
<th>ClogP</th>
<th>Dihedral Angle (deg)</th>
<th>Volume (Å$^3$)</th>
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<tbody>
<tr>
<td>2-hydroxy-2',3',4',5',6'-pentachlorobiphenyl</td>
<td>0.011</td>
<td>0.028</td>
<td>464</td>
<td>0.74</td>
<td>6.09</td>
<td>72</td>
<td>210.6</td>
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<tr>
<td>2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl</td>
<td>0.033</td>
<td>0.097</td>
<td>466</td>
<td>0.75</td>
<td>6.47</td>
<td>63</td>
<td>209.3</td>
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<td>2-hydroxy-2',3',4',5'-tetrachlorobiphenyl</td>
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<td>0.76</td>
<td>5.51</td>
<td>64</td>
<td>194.4</td>
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<tr>
<td>4-hydroxy-2',3',5',6'-pentachlorobiphenyl</td>
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<td>2.28</td>
<td>x</td>
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<td>6.19</td>
<td>60</td>
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<td>x</td>
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<td>6.39</td>
<td>70</td>
<td>213.8</td>
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<tr>
<td>4-hydroxy-2',4',6'-trichlorobiphenyl</td>
<td>2.50</td>
<td>15.4</td>
<td>472</td>
<td>0.74</td>
<td>5.09</td>
<td>66</td>
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<td>4-hydroxy-2',3',4',5'-tetrachlorobiphenyl</td>
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<td>17.5</td>
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<td>0.80</td>
<td>5.72</td>
<td>58</td>
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</tr>
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<td>4-hydroxy-3',3',4',5'-tetrachlorobiphenyl</td>
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<td>x</td>
<td>0.79</td>
<td>3.75</td>
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<tr>
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<td>4.14</td>
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ChemBio Office Software. The ClogP is a calculation of the $n$-octanol/water partition coefficient, log $P_{ow}$, which is a measurement of hydrophobicity of a molecule.

The Spearman correlation factors (statistical correlation between two variables) between the detection limits and the ClogP values, the dihedral angles and the molecular volumes were calculated, giving $R$ values of -0.87 ($P < 0.0001$), -0.66 ($P = 0.0071$) and -0.88 ($P < 0.0001$), respectively. These data revealed that there is indeed a correlation between the detection limit for an OH-PCB and the ClogP value, the dihedral angle and the molecular volume of that OH-PCB. These three parameters all contribute to the effect of the analyte on AEQ bioluminescence, albeit to a different degree. Depending on the analyte studied, one parameter will play a more important role than another, causing the differential responses observed. In general, the data in Table 4.1 indicates that the non-coplanar OH-PCBs that are more hydrophobic and have a greater volume demonstrate a higher affinity for the AEQ, which results in lower detection limits. Additionally, compounds having the hydroxyl group at the $ortho$ position seem to yield better responses than similar compounds with hydroxyl groups at the $para$ position. Two of the three coplanar OH-PCBs tested showed no effect on AEQ, while the third, 4-hydroxy-3,3’4’,5-tetrachlorobiphenyl, yielded only a small alteration of the bioluminescence signal. The ability of this compound to cause a decrease, although small, in the AEQ bioluminescence intensity, as compared to the ineffectiveness of the other two coplanar OH-PCBs, can be attributed to its higher hydrophobicity. The results obtained suggest that OH-PCBs that are non-coplanar, highly hydrophobic and occupy a larger molecular volume better inhibit aequorin activity. Specifically, we hypothesize that the OH-PCBs may co-exist with coelenterazine in the hydrophobic pocket of AEQ (see explanation below).

Since OH-PCBs are found in human blood,\textsuperscript{187-194} we aimed at demonstrating the feasibility of using this bioluminescence inhibition assay for detecting OH-PCBs in blood serum. For this, human serum samples were spiked with 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl standards at various concentrations prepared in DMSO. These spiked samples were added to apoAEQ and coelenterazine and, following the optimized
procedure described above, a dose-response curve was obtained (Figure 4.5). The detection limit for 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl in serum was calculated to be 5.02 x 10^{-6} M. The higher detection limit obtained in serum, compared to that obtained in buffer, can at least in part be attributed to the presence of human serum albumin (HSA). HSA is thought to bind to the OH-PCBs and therefore, would prevent the OH-PCB from interacting with the aequorin and inhibiting its bioluminescence. This is supported by the fact that a dose-response curve for 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl obtained using buffer containing HSA demonstrated a detection limit of 3.45 x 10^{-6} M, which is similar to that obtained using serum (data not shown).

A dose-response curve using tap water was also obtained to demonstrate the feasibility of employing this AEQ inhibition assay for detecting OH-PCBs in water samples. For this, tap water was spiked with the 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl standards at various concentrations prepared in DMSO, and a dose-response curve (Figure 4.5) was generated by employing the same assay conditions used for the response in buffer. The detection limit in tap water was found to be 1.32 x 10^{-7} M, which is comparable to the detection limit obtained in buffer, and the dose-response curve in tap water virtually overlapped to that in buffer, thus demonstrating that the matrix effect was negligible. In summary, we have demonstrated that it is feasible to detect OH-PCBs in biological and environmental samples by employing the AEQ inhibition assay that we developed. The concentrations of OH-PCBs in serum^{187-195,207,226} and in natural waters^{194,205,206} vary widely; therefore, there is a need for sensing systems able to detect these compounds at different concentrations in biological and environmental samples. In that regard, the rational tuning of AEQ can be employed to generate mutant AEQs with different properties and binding abilities, which would lead to shifts in the detection limits and working ranges, thus improving the analytical characteristics of the sensing system and its applicability.

The ability of other compounds to inhibit the bioluminescence of aequorin was investigated to evaluate the selectivity of the developed inhibition assay. For this, various
Figure 4.5. Dose-response curves for 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl obtained in buffer (red circles) as well as in serum (green triangles) and tap water (blue squares) by spiking serum or tap water with varying concentrations of the aforementioned OH-PCB and adding these samples to apoequorin and coelenterazine. The data points are an average of three measurements ± 1 standard deviation.
organic compounds were dissolved in DMSO, and then tested in the above assay format. The data in Figure 4.6 show that the compounds that are more hydrophilic than OH-PCBs, such as, amantadine, 3-chloro-benzoic acid and nicotine, do not decrease the bioluminescence generated by AEQ, which indicates that they are not binding to the protein. Therefore, they do not interfere with our inhibition assay. Only a few of the hydrophobic compounds tested demonstrate some inhibition of AEQ’s bioluminescence. However, the decrease in bioluminescence observed is not as pronounced as with 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl, indicating that these compounds have a low affinity for AEQ. The compounds 2,4,4’-trichlorobiphenyl, 2,2’dibromobiphenyl, pentachlorophenol and 2’,3,4,4’,5-pentachlorobiphenyl produce a 41%, 43%, 34% and 36% decrease in bioluminescence, respectively, whereas 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl at the same concentration produces a 91% decrease in bioluminescence when compared to the bioluminescence produced by AEQ in the absence of potential inhibitors. These four compounds are similar in structure to the OH-PCBs; therefore, it is reasonable to expect that AEQ would recognize them to a certain extent. Given that such compounds are also environmental pollutants, our system could be used as a screening system for a broader range of organic pollutants.

The inhibition of the AEQ bioluminescence by OH-PCBs is intriguing and could be occurring by any one of a number of mechanisms, including: (1) competitive inhibition, (2) uncompetitive inhibition, or (3) mixed inhibition. After examining the various possibilities and our data, we hypothesized that non-competitive inhibition, a form of mixed inhibition, occurs, thus allowing the OH-PCBs to co-exist in the hydrophobic pocket with coelenterazine. To investigate the validity of this hypothesis, we performed a series of structural and mechanistic studies. A common requirement for all three of the potential mechanisms of inhibition of AEQ bioluminescence is that the OH-PCB needs to bind to the protein. To confirm that the OH-PCB, 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl binds to AEQ, near-UV circular dichroism studies were performed. For this, apoaequorin was incubated with DMSO or 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl in DMSO for 15 min, and near-UV CD spectra were recorded. In addition, apoaequorin was first incubated overnight with coelenterazine to produce AEQ,
Figure 4.6. Selectivity studies performed by incubating a concentration of $1.23 \times 10^{-7}$ M apoAEQ with $1.0 \times 10^{-4}$ M concentrations of various compounds in the presence of $5.0 \times 10^{-10}$ M of coelenterazine. The data points are an average of three measurements $\pm 1$ standard deviation.
then the AEQ was incubated with DMSO or 2-hydroxy-2’,3’,4’5,5’-pentachlorobiphenyl in DMSO for 15 min, and near-UV CD spectra were obtained. Figure 4.7 shows that, as expected, 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl binds to apoaequorin (purple line), which is evident by the increase in the ellipticity with a broad peak at 290 nm observed. The addition of coelenterazine to apoaequorin caused a decrease in the molar ellipticity with sharp peaks at approximately 280 nm and 300 nm (blue line). Studies have established that certain ligands in the free (unbound) form have a small ellipticity signal, yet upon binding to a protein a large ellipticity signal is produced; this may help explaining why coelenterazine binding to apoAEQ results in a decrease of ellipticity, whereas OH-PCB binding to apoAEQ results in an increase in ellipticity.227-232 Specifically, both 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl and coelenterazine bind to apoaequorin and the difference in the changes in ellipticity, positive for 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl versus negative for coelenterazine, is attributed to the differences in chirality of the two ligands when bound to the protein. This CD data does not illustrate where 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl is binding to apoaequorin but it does prove that it binds to the apoprotein. Furthermore, the addition of 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl to AEQ caused the ellipticity to shift towards positive values. This implies that 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl is also binding when coelenterazine is present given that after fifteen minutes the spectra is shifting toward a positive ellipticity similar to when 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl binds to the apoprotein. It should be pointed out that in competitive inhibition the inhibitor cannot bind when the agonist is present, which is not the case here; however, from this CD data alone competitive binding cannot be ruled out because of the possibility of the OH-PCB binding to a site on the protein different from the coelenterazine’s binding site. In conclusion, the near-UV CD studies confirm that 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl binds to apoaequorin and AEQ, but the exact location of binding and the number of binding sites are yet to be determined.

To further prove that the OH-PCB binds to apoAEQ and to obtain a better understanding of where the binding may be occurring, tryptophan fluorescence studies were performed. Previous observations have shown that the intrinsic tryptophan
Figure 4.7. Near-UV CD spectra. The spectra were collected with a JASCO J-810 spectrometer (JASCO Ltd. UK) with 1 nm bandwidth and using a 1.0 cm pathlength cuvette. ApoAEQ with OH-PCB is in purple, ApoAEQ alone as a control is in green, AEQ with OH-PCB is in red and AEQ alone as a control is in blue. The wavelength scans were corrected for the blank and converted into molar ellipticity.
fluorescence of wild-type apoaequorin is quenched when coelenterazine is bound, allowing for the determination of an apparent $K_d$ of $1.2 \pm 0.12 \, \mu\text{M}$.\textsuperscript{210} This experiment was repeated with coelenterazine and 2-hydroxy-2′,3′,4′,5,5′-pentachlorobiphenyl and the cysteine-free apoaequorin. The data demonstrate that the 2-hydroxy-2′,3′,4′,5,5′-pentachlorobiphenyl quenches the tryptophan fluorescence in the same manner as coelenterazine; additionally, no red shift in the emission maximum, which is indicative of denaturation of the protein, is observed, thus suggesting that 2-hydroxy-2′,3′,4′,5,5′-pentachlorobiphenyl is not denaturing the protein but actually binding to the protein. The apparent $K_d$ of coelenterazine binding to the cysteine-free AEQ was found to be $1.4 \pm 0.12 \, \mu\text{M}$ (Figure 4.8), which is consistent with the reported value of $1.2 \pm 0.12 \, \mu\text{M}$ for the wild-type.\textsuperscript{210} The apparent $K_d$ of 2-hydroxy-2′,3′,4′,5,5′-pentachlorobiphenyl binding to the cysteine-free AEQ was found to be $0.78 \pm 0.11 \, \mu\text{M}$ (Figure 4.8). Surprisingly, even though the $K_d$ values are comparable, the 2-hydroxy-2′,3′,4′,5,5′-pentachlorobiphenyl has a higher binding affinity for apoaequorin than coelenterazine does. The intrinsic tryptophan fluorescence data suggests that the OH-PCB binds to the hydrophobic pocket as opposed to the exterior of the protein. In fact, AEQ contains 6 tryptophan residues, which all face the interior of the protein. Specifically, 5 of the 6 tryptophan residues are deep in the hydrophobic pocket and 3 of them are known to stabilize coelenterazine (Figure 4.9). Since the addition of the OH-PCB decreases the tryptophan fluorescence to such a great extent, and similar to the quenching observed when coelenterazine was added, we believe that the OH-PCB binds in the hydrophobic pocket as opposed to the exterior of the protein.

In view of the CD and tryptophan fluorescence data we hypothesize that 2-hydroxy-2′,3′,4′,5,5′-pentachlorobiphenyl binds to the hydrophobic pocket of AEQ, yet the mechanism of the bioluminescence inhibition has not been verified. In uncompetitive inhibition, the competitor binds to the protein-ligand complex. The CD and tryptophan fluorescence data both demonstrate that the OH-PCB binds to the protein without the ligand present, therefore the inhibition is assumed to be competitive or mixed. To further help elucidating the inhibition mechanism, the Gaddum/Schild equation can be employed.
Figure 4.8. Tryptophan fluorescence quenching. Each analyte (coelenterazine in red and OH-PCB in blue) was titrated into 1.22 µM apoAEQ. Each data point was blank subtracted and divided by the control (the appropriate solvent titrated into 1.22 µM apoAEQ to account for decrease in fluorescence intensity due to diluting the apoAEQ). The data points are an average of three measurements ± 1 standard deviation. The $K_d$ was calculated using binding-saturation fit in GraphPad Prism 5.0 (One Site-Specific Binding).
Figure 4.9. Coelenterazine (green) interaction with the amino acids in the 3 triads within the hydrophobic pocket of aequorin. Tyrosine, tryptophan and histidine are in red, blue and yellow, respectively.
as a guide to determine if an antagonist is behaving as a competitive or allosteric inhibitor, with allosteric inhibition being a form of mixed inhibition. A competitive antagonist yields a linear relationship between IC$_{50}$ values and the amount of antagonist with a Schild slope of 1. On the other hand, with allosteric antagonism the relationship between IC$_{50}$ values and the amount of the antagonist is hyperbolic. In order to investigate the nature of the antagonism, the concentration of the inhibitor, 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl, was kept constant at, above and below its IC$_{50}$ value, while the concentration of coelenterazine was varied (Figure 4.10). The data showed a linear relationship with a Schild slope of 1.28 ± 0.04 with an R$^2$ of 0.98. This data strongly suggests that competitive binding is occurring instead of an allosteric binding; however, a limitation of the Gaddum/Schild equation is that it cannot distinguish between competitive inhibition and non-competitive inhibition, a form of mixed inhibition. Therefore, further studies are needed to determine whether competitive inhibition (inhibitor binds to the receptor preventing the ligand from binding) or non-competitive inhibition (inhibitor binds to the receptor and reduces the activity of the protein but does not affect binding of the ligand) is occurring.

Generally, in competitive inhibition the presence of an inhibitor will cause an increase in K$_d$ while the B$_{max}$ is not altered; alternatively, in non-competitive inhibition in the presence of an inhibitor the K$_d$ is not altered, while the B$_{max}$ decreases. Therefore, the apparent K$_d$ and B$_{max}$ were determined for coelenterazine in the presence and absence of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl by evaluation of tryptophan fluorescence quenching. The data in Figure 4.11 demonstrates the effect of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl on coelenterazine binding to apoAEQ. The apparent K$_d$ without 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl is 1.14 ± 0.15 µM and the apparent K$_d$ with 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl is 1.29 ± 0.18 µM, showing that the K$_d$ is not altered; the B$_{max}$ without 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl is 0.90 ± 0.027, whereas the B$_{max}$ with 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl is 0.65 ± 0.023. Due to the unchanged app. K$_d$ and decrease in B$_{max}$ we believe the OH-PCB is acting as a non-competitive inhibitor, possibly co-existing in the hydrophobic pocket with coelenterazine.
Figure 4.10. Gaddum/Schild Response. A concentration of $1.23 \times 10^{-7}$ M apoAEQ was employed with varying concentrations of coelenterazine in the presence of a fixed concentration of 2-OH-2’,3’,4’,5,5’-pentachlorobiphenyl. No OH-PCB is in black, $1.0 \times 10^{-8}$ M OH-PCB is in green, $1.0 \times 10^{-7}$ M OH-PCB is in red and $1.0 \times 10^{-6}$ M OH-PCB is in blue. The data points are an average of three measurements ± 1 standard deviation. Plotted in GraphPad Prism 5.0 using Gaddum/Schild EC$_{50}$ shift non-linear fit.
Figure 4.11. Tryptophan fluorescence quenching. Coelenterazine was titrated into 1.22 µM ApoAEQ with (blue line) or without (red line) 1.22 µM 2-hydroxy-2’,3’,4’,5,5’-pentachlorobiphenyl. Each data point was blank subtracted and divided by the control (methanol titrated into 1.22 µM apoAEQ to account for decrease in fluorescence intensity due to diluting the apoAEQ). The data points are an average of three measurements ± 1 standard deviation. The $K_d$ and $B_{max}$ were calculated using binding-saturation fit in GraphPad Prism 5.0 (One Site-Specific Binding).
In order for coelenterazine and an OH-PCB to co-exist in the hydrophobic pocket, the pocket must have a large enough volume to contain both of them. Since the hydrophobic binding pocket of AEQ has a volume of 600 Å³, the combined molecular surface volumes of coelenterazine and the OH-PCB must be less than 600 Å³. The molecular surface volume of coelenterazine is calculated to be 363 Å³. The molecular surface volumes for each OH-PCB was calculated by using the minimized models of the corresponding OH-PCBs and the largest molecular volume was calculated to be 210 Å³ for 2-hydroxy-2’,3,4’,5’,6–pentachlorobiphenyl. Therefore, the largest combined volume is 577 Å³. This suggests that even the largest OH-PCB studied can co-exist in the pocket. Even though this seems like a tight fit, it is possible. In fact, there is a significant correlation between the detection limits and the molecular volumes (R = -0.88 (P < 0.0001), see above). We think that the larger the OH-PCB the more space it will occupy in the hydrophobic pocket, thus disrupting more of the interactions between coelenterazine and apoAEQ and producing greater inhibition of bioluminescent activity. Furthermore, the significant correlation between the detection limits and dihedral angles (R = -0.66 (P = 0.0071), see above) suggests that non-coplanar OH-PCBs have stronger interactions in the pocket and this could be due to the fact that non-coplanar molecules are less rigid and can adjust better in the hydrophobic pocket (Figure 4.1). Considering these mechanistic studies, we believe that the OH-PCBs are non-competitive inhibitors.

Even though further mechanistic studies are needed, another piece of evidence that favors non-competitive binding is the bioluminescence characteristics of AEQ in the presence of the OH-PCBs. Data illustrates that there are minor changes in the emission maxima and half-lives of AEQ in the presence of OH-PCBs compared to the emission maximum and half-life of AEQ in its native form (Table 4.1). Studies have shown that changing the amino acids in the binding pocket or replacing coelenterazine with its synthetic analogues can result in shifts in emission maximum or alter the half-life. These alterations in the bioluminescence characteristics are due to changes in the interactions that stabilize coelenterazine. Even though the changes in the emission spectrum and half-life are small, these changes indicate that interactions in the binding pocket are altered. If the OH-PCB was replacing coelenterazine, then there would be no change in these properties; therefore, the minor changes in the bioluminescence
characteristics of AEQ in the presence of OH-PCBs suggest that a non-competitive inhibition is plausible.

In conclusion, we have developed a novel AEQ-based bioluminescence inhibition assay for the detection of OH-PCBs. To the best of our knowledge this is the first time that AEQ has functioned as both the reporter and the recognition element for an analyte other than calcium. Except for calcium detection, the employment of AEQ as a reporter requires AEQ to be genetically fused or chemically conjugated to the analyte or recognition element. These steps are tedious, make the system more complex and may cause a decrease in AEQ bioluminescence, thus negatively affecting the attainable limits of detection. In our assay format, coelenterazine and the OH-PCB are incubated with apoAEQ and within two hours results are obtained. No separation steps are required or expensive instrumentation. Consequently, this assay can be employed as a rapid, cost-effective and easy-to-use screening method for OH-PCBs. In addition, the feasibility of detecting OH-PCBs in spiked biological and environmental samples was confirmed. It should be pointed out that rational tuning of AEQ can be accomplished to develop mutant AEQs, which would be employed in analogous bioluminescence inhibition assays leading to shifts in the detection limits and working ranges, thus improving the analytical characteristics of the sensing system and its applicability. The rational tuning of AEQ has been studied demonstrating that changing various amino acids has created mutants with different properties.\(^{236-238}\) Given that the OH-PCBs are binding in the hydrophobic pocket, altering amino acids in the pocket could change the interactions between the protein and OH-PCB, thus changing the analytical performance characteristics of the inhibition assay accordingly. Further, our findings have demonstrated a unique approach to using AEQ as a reporter as well as a recognition element allowing for an original class of AEQ inhibition assays to be investigated. This platform could be used to screen a variety of molecules depending on their interactions with AEQ and has provided insight to interferences that can disrupt the bioluminescence of AEQ in nature.
CHAPTER FIVE

EXPLORING THE TRUNCATION OF AEQUORIN FOR BIOANALYTICAL APPLICATION

Introduction

Nature has bestowed upon us many biomolecules that are being investigated through fundamental and applied research. Bioluminescent proteins are one category of such biomolecules provided by nature. Bioluminescence is a rare, natural phenomenon in which visible light, produced by a biochemical reaction, is emitted by an organism. The calcium-dependent photoprotein aequorin (AEQ) is a well-studied bioluminescent protein that was isolated in 1961 from the jellyfish *Aequorea victoria*. The aequorin molecule consists of apoaequorin (apoAEQ), which is a polypeptide 189 amino acids long, molecular oxygen and the organic chromophore, coelenterazine. Aequorin is a 21.4 kDa protein that contains four EF-hands, or helix-loop-helix domains, three of which bind calcium. In the presence of calcium, aequorin undergoes a conformation change and the coelenterazine is converted into excited coelenteramide and CO₂. The return of the excited coelenteramide to the ground state is accompanied by a flash of blue light at 469 nm.

Aequorin has a number of advantages over other molecules when used as a reporter for detection of target analytes. Since bioluminescence is the result of a biochemical reaction no excitation source is needed for the photoprotein to emit light, differently from fluorescence. For this reason, there is no background light deriving from concomitant excitation of other autofluorescent compounds present in a sample, which facilitates lower detection limits. Furthermore, AEQ can be detected at exceptionally low levels, in the attomole range. AEQ is biologically harmless and therefore, it can be employed for detection in vivo. In addition, AEQ can be detected over a broad dynamic range using a standard inexpensive luminometer. Because of these features, AEQ has been employed in a variety of bioanalytical applications, including as a calcium reporter in living cells, for in vivo imaging, as well as a reporter in DNA hybridization.
assays\textsuperscript{62,216-218} and immunoassays.\textsuperscript{47,219-223} The gene of apoaequorin has also been subjected to site-directed mutagenesis resulting in variants of AEQ having different emission maxima and half-lives, which has allowed for the protein to be used in assay systems that can detect multiple analytes simultaneously.\textsuperscript{244} Furthermore, AEQ has been used as the signal generating molecule in protein switch sensing systems\textsuperscript{224,225} and as a reporter for a protein truncation test.\textsuperscript{245}

To further expand the utility of AEQ, we have begun exploring rationally designed truncated variants of AEQ for bioanalytical applications. Truncated AEQs have the potential to be employed as a reporter for multi-analyte detection and genetic tagging, protein tagging or protein-protein interactions depending on whether the aequorin fragments are bioluminescent alone, they self-assemble in the presence of coelenterazine or they only assemble into an active unit in the presence of interacting partners, respectively (Figure 5.1). Bioluminescent labels are ideal for multi-analyte detection since they can be genetically modified to have different spectral and/or decay properties.\textsuperscript{246} Thus, the signals of the modified labels can be distinguished from one another while still providing sensitive detection. Spectral tuning of AEQ has been carried out by changing the bioluminescence characteristics of the photoprotein through a combination of site-directed mutagenesis and the use of coelenterazine analogues.\textsuperscript{246} Recently, researchers have shown that an AEQY82F mutant paired with coelenterazine has an emission maximum of 519 nm, whereas an AEQW86F mutant paired with coelenterazine hcp has an emission maximum of 445 nm. With these two pairs there is a 74 nm difference in the emission peaks. As a result, they can be used together for wavelength resolved dual-analyte detection.\textsuperscript{236} In addition, Dikici et al. produced aequorin variants with half-lives ranging from 0.23 to 50.1 seconds; accordingly, these variants can be used for time resolved dual-analyte detection.\textsuperscript{236} Even though AEQ variants with different emission maxima and half-lives are currently available, there is still a need for expansion of the spectral diversity of AEQ. Variants with broader spectral and decay characteristics could enable the detection of more than two analytes simultaneously. In addition, finding an AEQ mutant that is more red-shifted and/or has a longer half-life could prove to be a
Figure 5.1. A and B represent two AEQ fragments. The red sphere is coelenterazine. a.) Auto-luminescence AEQ fragments. b.) Self-assembled AEQ fragments for protein tagging. c.) Assisted-assembled AEQ fragments – protein interaction partner 1 (PPI1) must interact with protein interaction partner 2 (PPI2) for the AEQ fragments to come together and emit bioluminescence.
valuable tool for in vivo imaging given that red light is more suitable for deep tissue imaging and long stable signals are more appropriate for three-dimensional imaging.

The development of truncated AEQ fragments that are active could lead to a larger array of useful AEQ-derived reporters. It is possible that removing portions of the apoprotein will change the interactions between the apoprotein and coelenterazine, creating proteins with different emission maxima and/or half-lives, which could be employed in multi-analyte detection or in vivo imaging. In addition, these truncated proteins could be utilized as genetic tags. A limitation of current genetic tags is the size of the tag; large tags can interfere with the function of the protein of interest. We envision the possibility of designing small truncated functional AEQ fragments which can overcome this limitation.

Protein tagging systems are used to monitor protein solubility and folding in vivo and can be used to assay promoters of protein folding or inhibitors of protein aggregation. For example, fragments of β-galactosidase have been employed as protein tags to monitor the solubility of proteins, including the Alzheimer’s Aβ (1-42) peptide. Another study has shown that fragments of the green fluorescent protein (GFP) can self-assemble and be employed as a tag for the detection of soluble or insoluble proteins. The β-galactosidase system is limited because it can decrease protein solubility due to poor folding and the fluorescent system is limited by light scattering and source instability. Protein tagging using AEQ involves discovering truncated AEQ fragments that alone do not auto-bioluminesce, but will self-assemble with a complementary truncated AEQ fragment, in the presence of coelenterazine, to form an active unit. The availability of self-assembling AEQ fragments would open up a new avenue for employing aequorin as a protein tag, which could overcome some of the limitations of current systems and expand the protein tagging toolbox.

Protein-protein interactions (PPIs) play a central role in nearly all cellular processes. The ability to monitor PPIs has been and continues to be vital to understanding, elucidating and mimicking cellular processes, given that the majority of
proteins work together in complex pathways. Existing methods for monitoring PPIs include mammalian or yeast two hybrid systems,\textsuperscript{116,117} protein complementation assays (PCA),\textsuperscript{118} protein microarrays,\textsuperscript{119} mammalian protein-protein interaction trap (MAPPIT),\textsuperscript{120} luminescence-based mammalian interactome (LUMIER),\textsuperscript{121} immunoprecipitation,\textsuperscript{122} affinity purification coupled with mass spectrometry,\textsuperscript{123} fluorescence resonance energy transfer (FRET)\textsuperscript{124} and bioluminescence resonance energy transfer (BRET).\textsuperscript{125} Most of these methods suffer from various drawbacks, thus having limitations. An ultimate goal of detecting PPIs is to determine the location and dynamics of interactions \textit{in vivo}. Two hybrid systems, PCAs and MAPPIT as well as BRET and FRET are methods that can detect PPIs \textit{in vivo}. However, the two hybrid systems and the MAPPIT system are limited because they only work in the nucleus or cytosol, respectively. BRET and FRET are successful techniques; nevertheless, the analysis is fairly complex, high expression levels of proteins are required and energy transfer from non-specific interactions can interfere.\textsuperscript{128} On the other hand, the PCA technique shows the most promise given that it has the ability to monitor the location and dynamics of PPIs, as well as inhibition of PPIs, \textit{in vivo}. PCAs involve splitting a reporter protein into two non-functional fragments and genetically linking these two fragments to protein interaction partners. When the partners interact, the fragments of the split reporter will come into contact and form an active unit. Presently, there are only seven reporters, dihydrofolate reductase, β-lactamase, TEV protease, β-galactosidase, ubiquitin, green fluorescent protein and its variants and the luciferases, most commonly \textit{renilla} and firefly, that have been employed in PCAs.\textsuperscript{118} Of these seven, fluorescent and bioluminescent reporters are more applicable for studying the location and dynamics of a particular interaction due to their spectroscopic nature. Fluorescent reporters are more suitable for localization of PPIs since there is no need for a substrate, whereas bioluminescence reporters are more appropriate for PPI dynamics provided that their reassembly is reversible due to the presence of independent sub-domains in the protein.\textsuperscript{118} To that end, the rational design and characterization of AEQ reporter pairs that could be employed in PCAs would provide an alternative reporter for PCAs and the study of PPIs.
Herein, we will tell our story of how the development of a caspase-3 protein switch sensing system led to studies of the truncation of AEQ. In addition, we report the current results and speculate on the future of truncated AEQs. The potential applications of truncated AEQ considered here are for multi-analyte detection, genetic tagging, protein tagging and monitoring protein-protein interactions. The rigid structure of AEQ was studied and fragments were rationally designed. Presently, one truncated fragment has been characterized. The bioluminescent properties of the additional fragmented AEQs should not only be useful analytically, but could help us better understand some of the properties of the mechanism responsible for the bioluminescence emitted by AEQ.

**Materials and Methods**

**Materials**

Tris free base – Tris(hydroxymethyl)amino methane was purchased from Serva (Heidelberg, Germany). Glycine, sodium chloride, disodium ethylenediaminetetraacetate (EDTA), Luria-Bertani (LB) Agar and LB Broth were purchased from Fischer Scientific (Fair Lawn, NJ). Albumin from bovine serum (BSA), protein-A alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate disodium salt, nitrotetrazolium blue chloride, dimethyl sulfoxide (DMSO) anhydrous, calcium chloride, ampicillin sodium salt, chloramphenicol, kanamycin monosulfate and ethidium bromide were purchased from Sigma-Aldrich (St. Louis, MO). Methanol was purchased from EMD Biosciences (Darmstad, Germany). Imidazole was purchased from J.T. Baker. (Phillipsburg, NJ). Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Gold Biotechnology, Inc. (St. Louis, MO). Sodium Dodecyl Sulfate (SDS) was purchased from Curtin Mathesin Scientific, Inc. (Houston, TX). The Bradford protein assay kit was purchased from Bio-Rad Laboratories. (Hercules, CA). Ni-NTA Agarose, mini-prep kit and gel extraction kits were purchased from Qiagen (Valencia, CA). Caspase-3 and the caspase-3/CPP32 Colorimetric Assay Kit were purchased from Biovision (Mountain View, CA). Rabbit polyclonal primary antibody to AEQ (Catalogue # ab9096) was purchased from Abcam (Cambridge, MA). Top10 chemical competent cells, Tris glycine SDS Page gels and PVDF membranes were purchased from Invitrogen (Carlsbad, CA). The plasmid, pET41(a)+, BL21(DE3) chemical competent cells and recombinant enterokinase kit were
purchased from Novagen (Madison, WI). Gel code blue stain and 3,500 MWCO 3-12 mL Slide-A-Lyzer dialysis cassettes were purchased from Pierce (Rockford, IL). G-Bioscience Tube-O-Dialyzers were purchased from VWR International (Bridgeport, NJ). T4 DNA ligase, alkaline phosphatase and Eco RI, Hind III and Nhe I restriction enzymes were purchased from Promega (Madison, WI). Pfu Ultra polymerase and Taq polymerase were purchase from Strategene (Ceder Creek, TX). All primers for PCR were purchased from Operon (Huntsville, AL). All chemicals were reagent grade or better and solutions were prepared using deionized reverse osmosis (Milli-Q Water Purification System, Millipore, Bedford, MA) water.

Apparatus
Polymerase chain reactions (PCR) were performed on an Eppendorf Mastercycler Personal thermocycler (AG, Hamburg). DNA electrophoresis was performed using a FB105 Fischer Biotech Electrophoresis Power Supply (Pittsburg, PA) and the gels were visualized using a UV Transilluminator (UVP, Upland, CA). OD$_{600}$ readings were taken using a Spectronic 21D (Milton Roy, Ivy Land, PA). Cells were sonicated using a Fischer Scientific 550 Sonic Dismembrator (Pittsburg, PA). Proteins were expressed by incubating bacteria at 37°C on a Forma Scientific Orbital Shaker (Waltham, MA) and harvested using a Beckman J2MI centrifuge (Palo Alto, CA). Proteins were purified using Qiagen Ni-NTA resin (Valencia, CA). Purity of the proteins were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Invitrogen 10-20% Tris-glycine gels in an Invitrogen X Cell Sure Lock Mini Cell (Carlsbad, CA). Western Blots were done using Invitrogen PVDF membranes in an Invitrogen X Cell II Blot Module (Carlsbad, CA). OD 280 readings were taken using an Agilent 8453 UV-Visible Spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA). Bioluminescence measurements were made on an Optocomp I test tube luminometer (MGM Instruments, Inc., Hamden, CT) using Fischer borosilicate 12 x 75 mm disposable glass tubes (Pittsburgh, PA). Bioluminescence emission spectrum was measured on a Spectro System Spectrographic Luminometer custom built by Sciencewares, Inc. (Falmouth, MA). Bioluminescence half lives were measured on a FLUOstar Optima luminometer (BMP Labtech, Cary, NC).
Methods – Caspase-3 Recognition Site (Asp-Glu-Val-Asp; DEVD)-AEQ Fusion Protein

Construction of Plasmid p47DEVDAEQ. The apoaequorin cysteine-free mutant gene was used as the template for PCR. The following primers were designed to obtain the gene fragments, which encode for aequorin amino acids 1-47 and aequorin amino acids 48-189:

1) GAGGCTAGCGTGAAACTGACCAGCGACTTCGACAACCCAAGATGG
2) TGGCTCAGGTGTTGCTCCAAGATCCCACCTCATCGTTATTGATGACAATATCAGA
3) CACAGGCTTTTAGGGGACACGCTCCACCCTAGAGCTTTTCGGAAGCAGGATC
4) GATGAAAGTGGATCTTGGAGCAACACCTGAGC

Primers 1 and 2 were used to amplify the apoaequorin sequence coding for amino acids 1-47. Primer 1 introduced an Nhe I restriction site (underlined) on the 5’ end of the coding sequence, and primer 2 introduced the caspase-3 recognition site (in Bold; it codes for the amino acids Asp-Glu-Val-Asp) on the 3’ end. Primers 3 and 4 were used to amplify the aequorin sequence coding for amino acids 48-189. Primer 3 introduced a Hind III site (underlined) on the 3’ end and primer 4 introduced the caspase-3 recognition site on the 5’ end. The PCRs were performed using the Pfu Ultra polymerase employing 30 cycles with denaturing, annealing and elongation conditions of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 2 minutes, respectively. The apoAEQ1-47 and 48-189 gene sequences amplified above were then used as templates in an overlap PCR. Primers 1 and 3 from above were used in this PCR. This resulted in a DNA sequence coding for AEQ containing an Nhe I site on the 5’ end and a Hind III site on the 3’ end with the coding sequence for the caspase-3 recognition site between amino acids 47 and 48. The overlap PCR product was then subcloned into the pCR2.1-TOPO vector, using the TOPO TA Cloning method by Invitrogen. Plasmid DNA was isolated using the Qiagen Mini
Prep Kit (Valencia, CA). The 47DEVDAEQ DNA obtained by TA cloning was digested with Nhe I and Hind III restriction enzymes. The plasmid, pET28(a)+, was also digested with Nhe I and Hind III. The digested 47DEVDAEQ DNA insert was ligated into pET28(a)+ yielding p47DEVDAEQ. The p47DEVDAEQ vector was transformed into E. coli BL21(DE3) cells, and the DNA was isolated for sequencing. The constructed DNA was sequenced at the University of Kentucky Advanced Genetics Technology Center (AGTC) to confirm the in frame DNA sequence of apoAEQ1-47-DEVD-apoAEQ48-189.

**Construction of Plasmid p24DEVDAEQ.** The vector p24DEVDAEQ was constructed as described above for p47DEVDAEQ except the following primers were used in place of primers 2 and 4:

5) GTCAAGAGAGATTTTTCCATTATCCACTTCATCAAGGAAATTGAA
   CATATGCCTT

6) GATGAAAGTGGATAATGGAAAAATCTCTCTTTGAC

Once fully constructed p24DEVDAEQ contained a gene sequence of apoaequorin, where the residues Glu-Val-Glu replace the amino acid residues at positions 25-27 of the native apoaequorin sequence. An Nhe I site was on the 5’ end and a Hind III site on the 3’ end of the insert DNA, just as above. This was also verified by DNA sequencing at AGTC.

**Expression and Purification of 24DEVDAEQ and 47DEVDAEQ fusion proteins.**
Expression using p24DEVDAEQ and p47DEVDAEQ results in apoaequorins with 6xHis-tags on the N-termini for easy purification. The p24DEVDAEQ and p47DEVDAEQ plasmids were transformed into chemically competent E. coli BL21(DE3)pLysS cells. The method for growth of the cells and purification of the fusion proteins was the same for 24DEVDAEQ and 47DEVDAEQ. The bacteria cells were grown overnight, at 37 °C in an orbital shaker at 250 rpm, in 5 mL of Luria Bertani (LB) broth containing 30 μg/mL kanamycin and 34 μg/mL chloramphenicol. The overnight culture was used to inoculate 500 mL of LB broth containing 30 μg/mL kanamycin and 34 μg/mL chloramphenicol and the cells were grown at 37 °C at 250 rpm. When the
culture reached an OD_{600} of 0.4, IPTG was added to 1.0 mM final concentration, and the culture was allowed to grow for 2 hours. The cells were harvested by centrifugation at 22,100 x g at 4 °C for 15 minutes. The bacterial pellet was resuspended in 50 mM NaH_{2}PO_{4}, pH 8.0, 300 mM NaCl, 10 mM imidazole buffer (NiNTA Lysis buffer) and sonicated on ice using 10 second burst followed by 10 second rest for 10 minutes total using a Fischer Scientific 550 Sonic Dismembrator (Pittsburg, PA). The suspension was centrifuged at 11,100 x g, at 4 °C, for 20 minutes to obtain the supernatant containing the 6xHis-tagged fusion protein.

The proteins were purified using immobilized metal affinity chromatography (IMAC) using a Nickel-NitriloTriAcetic acid (Ni-NTA) Agarose column. Ni-NTA agarose was added to the supernatant and it was agitated at 4 °C for 1 hour. Then, the supernatant containing the Ni-NTA Agarose resin was poured into a gravity flow column. The resin was washed two times with 50 mM NaH_{2}PO_{4}, pH 8.0, 300 mM NaCl, 20 mM imidazole buffer. Following the wash, protein was eluted using 1.5 mL aliquots of 50 mM NaH_{2}PO_{4}, pH 8.0, 300 mM NaCl, 250 mM imidazole buffer. SDS-PAGE was run to verify which fractions contained the fusion protein. Fractions containing the fusion protein were pooled together and an OD_{280} reading was taken. Protein concentrations were calculated using an extinction coefficient of 43,430 M^{-1} cm^{-1}.

24DEVDAEQ and 47DEVDAEQ – Caspase-3 Assay. A final concentration of 1μg/mL coelenterazine was added to each fusion protein, and these solutions were incubated overnight at 4 °C. To each 1 ml aliquot of the substrate solutions, 2X caspase-3 reaction buffer (50 mM Hepes, pH 7.2, 50 mM NaCl, 0.1% Chaps, 10 mM EDTA, 5% Glycerol, and 10 mM DTT) and a two times molar excess of 1 U/μl Caspase-3 was added. At the same time, control reactions that contained the 24DEVDAEQ and 47DEVDAEQ substrates, alone, were set up in 2X reaction buffer. A positive colorimetric control was set up using the Biovision DEVD-pNA colorimetric substrate in the presence of the 2X reaction buffer and caspase-3. These were left at room temperature for 96 hours, and at different times 10 μl of the reactions were checked for bioluminescence on the Optocomp.
I. The procedure to check the bioluminescence activity is described in the following section.

Bioluminescence Emission Study. An aliquot of ten microliters of protein was assayed in an Optocomp I Test Tube Luminometer (MGM Instruments, Inc., Hamden, CT). Bioluminescence was triggered by injecting 50µL of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl₂ (Triggering Buffer). The bioluminescence signal was integrated for 6-s, and reported in Relative Light Units (RLU’s). The Optocomp I instrument has a photomultiplier tube used for the detection of photons which are converted to electrical pulses. The number of pulses counted is proportional to the bioluminescent light emitted. This is displayed in Relative Light Units (RLU’s).

Western Blot. Native SDS-PAGE was run using standard protocols with the Benchmark pre-stained ladder (Invitrogen). The proteins were transferred onto a PVDF membrane in 1X Novex Tris Glycine Transfer buffer (Invitrogen) with 20% methanol for 1 hour at 25 volts. The membrane was blocked with a blocking solution, 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10% (w/v) nonfat dry milk, for 30 minutes at room temperature while shaking gently. The blocking solution was removed and the primary antibody (Rabbit polyclonal to AEQ) diluted 1:1000 with the blocking solution was added to the membrane. The primary antibody was incubated for one hour while gently shaking at room temperature and then removed. The membrane was washed three times, for ten minutes, with 15 mL of 20 mM Tris/HCl, pH 7.5, 150 mM NaCl buffer. The secondary antibody, protein A-alkaline phosphatase, diluted 1:2000 with the blocking solution, was added to the membrane and incubated at room temperature for one hour while shaking gently. The secondary antibody was removed and the membrane was washed three times, for ten minutes, with 15 mL of 20 mM Tris/HCl, pH 7.5, 150 mM NaCl buffer. The membrane was washed a final time with 15 mL of 100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂ buffer at room temperature for ten minutes. For the alkaline phosphatase substrate solution 100 μLs of 20 mg/mL 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) in dimethyl formamide (DMF) and a 50 mg/mL
nitrotetrazolium blue chloride (NTB) in DMF were added to 20 mL of 100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂ buffer. The substrate solution was added to the membrane and incubated with gentle shaking at room temperature until the blot was developed. After the desired contrast for the protein bands were reached, the development of the membrane was quenched using DI water and the membrane dried.

Method – Truncated Proteins

Construction of Plasmid pAEQ1-47. The apoaequorin cysteine-free mutant gene was used as the template for PCR. The following primers were designed to obtain the gene which encodes for aequorin amino acids 1-47:

7.) GAGGAATTC
    GTGAAACTGACCAGCGACTTCGAC
8.) CACAAGCTTTTAGTTATTGATGACAATATCAGA

Primer 7 was used to introduce an Eco RI restriction site (underlined) on the 5’ end and primer 8 was used to introduce a Hind III site (underlined) on the 3’ end of the insert. The AEQ1-47 PCR product was subcloned in the pCR2.1-TOPO vector, using the TOPO TA Cloning method by Invitrogen. Then the plasmid was isolated using the Qiagen Mini Prep Kit. The DNA insert obtained by TA cloning and the vector, pET41(a)+ were digested using Eco RI and Hind III restriction enzymes. Then, the digested apoaequorin DNA insert was ligated into pET41(a)+ yielding the plasmid pAEQ1-47. DNA sequencing was performed at the AGTC to confirm the DNA sequence of apoaequorin amino acids 1-47.

Construction of Plasmid pAEQ48-189. This plasmid was constructed as described above for pAEQ1-47 except the following primers were used:

9.) GAGGAATTC TTCCTTTGGAGCAACACCTGAGCAAGCC
10.) CACAGGCTTTTAGGGGACAGATCCACCGTAGAGCTTTTCGGAAGC

AGGATC

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DNA sequencing at the ATGC confirmed an in frame gene sequence of apoaequorin amino acids 48-189 with an Eco RI restriction site on the 5’ end and a Hind III site on the 3’ end.

**Expression and Isolation of Truncated Proteins.** The pAEQ1-47 and pAEQ48-189 plasmids were transformed into chemically competent *E. coli* BL21(DE3) cells. The method for growth of the cells and purification of the fusion proteins was the same for cells containing pAEQ1-47 and pAEQ48-189. The bacteria were grown overnight in 5 mL of LB broth containing 30 μg/mL kanamycin at 37 °C in an orbital shaker at 250 rpm. Then, the culture was used to inoculate 500 mL of LB broth containing 30 μg/mL kanamycin and grown at 37 °C at 250 rpm. When the culture reached an OD\textsubscript{600} of 0.6, IPTG to a final concentration of 1 mM was added and the bacteria were incubated at 37 °C for 2 hours. The culture was harvested by centrifugation at 22,100 x g, at 4 °C, for 15 minutes. SDS-PAGE was run to verify expression of the truncated proteins. Ni-NTA purifications were performed for each truncated protein as described above. SDS-PAGE was run to verify which fractions contained the truncated proteins and the fractions containing the proteins were pooled together. Each suspension containing AEQ1-47 or AEQ48-189 was treated in the same manner for the purification of the proteins. The suspension containing the truncated protein was dialyzed overnight against 2 L of recombinant enterokinase cleavage buffer (200 mM Tris/HCl, pH 7.4, 500 mM NaCl, 20 mM CaCl\textsubscript{2}) using 3500 MWCO Pierce dialysis cassettes. The concentration of protein was determined by OD\textsubscript{280} and 1 unit of recombinant enterokinase was added for every 50 μg of protein. The enterokinase cleavage reaction was incubated at 4 °C for 48 hours on a rotary mixer. SDS-PAGE was run to verify the presence of the cleavage and the enterokinase was removed from the reaction mixture using the recombinant enterokinase cleavage capture protocol (Novagen). A Ni-NTA purification was performed as described above then SDS-PAGE was run to confirm the purity of the proteins. The truncated fragment was denatured by adding 6 M urea and leaving the suspension to rotate at 4 °C overnight. The denatured, truncated protein was refolded as follows: the protein was dialyzed sequentially against 1.) 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 4 M urea buffer,
Bioluminescence Emission Study. A three times molar excess of native coelenterazine was added to the truncated protein and the mixture was left at 4 °C for 18 h. Ten microliters of protein were assayed in an Optocomp I Test Tube Luminometer (MGM Instruments, Inc., Hamden, CT). Bioluminescence was triggered by injecting 50µL of the Triggering Buffer. The bioluminescence signal was integrated for 6-s. And the bioluminescence signal was measured in Relative Light Units (RLU’s).

Bioluminescence Emission Spectra. The emission maximum was determined in the presence of the following coelenterazine analogues: native, i, cp, f, fcp, h, ip, n, and hcp. A three times molar excess of the each coelenterazine analogue was added to the truncated protein and the mixture was incubated at 4 °C for 18 h. To determine the emission maximum, 25 µl of the truncated protein was added to three separate wells of a microtiter plate. Bioluminescence emission was triggered by injecting 50 µL of the Triggering Buffer and the bioluminescence signal was collected with a cooled CCD camera between the wavelengths of 400 and 1200 nm on a Spectro System Spectrographic Luminometer custom built by Sciencewares, Inc. (Falmouth, MA).

Bioluminescence Half Life Study. The half-lives of the bioluminescence reaction for the truncated proteins were determined in the presence of the following coelenterazine analogues: native, i, cp, f, fcp, h, ip, n, and hcp. A three times molar excess of the each coelenterazine analogue was added to the truncated protein and the mixture was incubated at 4 °C for 18 h. To determine the half life, 25 µl of the truncated protein was added into the wells of a microtiter plate in triplicates. Bioluminescence reaction was
triggered by injecting 100 µL of Triggering Buffer, and the photons were collected using FLUOstar Optima luminometer for 6-s and the half-lives were calculated by fitting the decay data to a one phase exponential decay kinetics equation in GraphPad Prism 5.0 software.

**Circular Dichroism (CD).** The stock solution of truncated apoAEQ was dialyzed overnight against 2 L of a low salt buffer (5 mM phosphate, pH 7.0, 10 mM EDTA). The concentration of the protein was determined by measuring the absorbance at 280 nm (AEQ48-189 has an extinction coefficient of 36,440 M⁻¹ cm⁻¹ at this particular wavelength). The truncated apoprotein was then incubated with native coelenterazine, to a final concentration of 1µg/mL, overnight at 4 °C. The CD spectra were collected with a JASCO J-810 spectrometer (JASCO Ltd. UK) with a 1 nm bandwidth using a 0.1 cm pathlength cuvette for the far UV scans. The wavelength scan was performed at 20 °C, and corrected for the blank. The blank was composed of the low salt buffer and coelenterazine. The same procedure was followed for full length cysteine free AEQ which was used for comparison.

**Results and Discussion**

Aequorin is a photoprotein that has been employed as an extremely sensitive label in a variety of sensing systems and as a calcium indicator. Genetic engineering of aequorin continues to expand its applications as a bioanalytical tool. Originally, we proposed to develop a protein switch sensing system selective for caspase-3 employing split AEQ as the reporter. Caspase-3 is a protease involved in regulating apoptosis. The aequorin based switch sensing system was intended to lead to an extremely sensitive, homogeneous assay for caspase-3 that could be employed to identify caspase-3 inhibitors as potential drug candidates. Our caspase-3 protein switch system was proposed to function in such a way that the caspase-3 recognition site, Asp-Glu-Val-Asp (DEVD), was inserted into the amino acid sequence of apoAEQ so that the DEVD insertion would not disrupt the bioluminescence of AEQ. However, in the presence of caspase-3 the
modified AEQ substrate would be cleaved in two fragments leading to a loss of bioluminescence.

The rational protein engineering design for preparation of the AEQ protein switch can be found in Hamorsky, et al. Briefly, the modified AEQ substrate was rationally designed to have the amino acids DEVD inserted in between amino acids 47 and 48 of a cysteine free mutant of AEQ. This location was selected because the amino acid residues 47 and 48 are in an outer loop between EF-hand I and II of AEQ (Figure 5.2). Therefore, we hypothesized that the insertion of DEVD would not interfere with calcium or coelenterazine binding; additionally, given that the DEVD sequence will be inserted in an outer loop we theorized that it will be accessible for caspase-3. For this, the PCR was used to create the gene sequence encoding for apoaequorin amino acids 1 through 47, followed by the DEVD caspase recognition site and then apoaequorin amino acids 48 through 189 (1-47apoAEQ-DEVD-48-189apoAEQ). The protein resulting from the expression of this sequence is referred to as 47DEVDapoAEQ. The purified 47DEVDapoAEQ protein was incubated overnight at 4 °C with coelenterazine. The protein with coelenterazine bound, referred to as 47DEVDAEQ, was found to have a specific activity of 2.47 x 10^9 RLU/mg. The cysteine free mutant of aequorin has a bioluminescence of aequorin, specific activity in the order of 10^{12} RLU/mg; therefore, the modification did have some effect on the but the substrate (modified AEQ) was active enough to be employed for the detection of caspase-3.

To test the ability of this modified AEQ to function as a protein switch sensing system for detection of caspase-3, the purified 47DEVDapoAEQ was incubated with coelenterazine, and then a two times molar excess of caspase-3 was added. The 47DEVDAEQ without addition of the caspase-3 was employed as a control. Incubations were performed at room temperature and the bioluminescence signals were measured at various incubation times. Additionally, a colorimetric substrate for caspase-3 was used as a control of enzyme activity. The colorimetric substrate changed from colorless to yellow within an hour of adding the caspase-3, verifying an active caspase-3 enzyme. However,
Figure 5.2. The 3-D structure of aequorin. Protein databank structure 1EJ3. EF-hand I is in blue, EF-hand II is in green, EF-hand III is in yellow and EF-hand IV is in red. Visualized using Discovery Studio Visualizer 2.5.5.
as can be seen from the data in Table 5.1, the addition of caspase-3 to 47DEVDAEQ (column 2) did not cause a loss in bioluminescence that was largely different from the control (column 3). These results suggested that either the 47DEVDAEQ is not a proper substrate for caspase-3 or the enzyme is cleaving the DEVD site, but the protein is not losing bioluminescent activity. To determine which of these hypotheses is correct, the caspase-3 / 47DEVDAEQ reaction mixture (after 96 h incubation) was subjected to affinity chromatography using a Ni-NTA agarose resin column, as described in the Experimental Section for protein purification. A His-Tag is present on the N-terminus of the 47DEVDAEQ; therefore, if the protein is cleaved by caspase-3 the C-terminal fragment will not bind to the nickel agarose and would be found in the flow through fraction, while the N-terminal fragment will bind to the nickel and be present in the elution fractions. The flow through and elution fractions were analyzed by SDS-PAGE and the presence of the truncated proteins was confirmed by western blot analysis (Figure 5.3a). If the 47DEVDAEQ substrate is cleaved by caspase-3, the N-terminal end should produce a band at 7,982 Da and the C-terminal fragment should produce a band at 15,895 Da. The western blot analysis verified that the 47DEVDAEQ protein is a substrate for caspase-3 because a band at approximately 16 kDa was observed in the flow through fraction (Figure 5.3a). However, no band at 7,982 Da was observed in the elution fractions. This could be either due to the lack of an epitope on apoAEQ amino acids 1-47 for binding to the antibody in the western blot or to the fragment being too small to be seen on the particular type of gels that were used. These results suggest that the caspase-3 cleaved the 47DEVDAEQ although the cleavage did not result in a measurable loss in bioluminescence that was significantly different than the control.

Since cleavage of the 47DEVDAEQ did not result in a loss of aequorin activity, a new site was rationally selected to insert DEVD into apoAEQ, with the aim to create a protein switch capable of detecting caspase-3 and usable for the screening of its inhibitors. We hypothesized that inserting DEVD into a calcium binding site could result in a functional switch because, when cleaved, the calcium binding site would no longer bind calcium and, as a result, aequorin activity would be lost or decreased. At the same time, the DEVD insertion must allow AEQ to remain active before cleavage. Study of the
Table 5.1. Effect of caspase-3 on the bioluminescence of AEQ-based substrates. Bioluminescence (total RLUs) was measured at each incubation time period. The data in the table represent the percentage of bioluminescence left at each time point calculated as (total RLUs at each time point / total RLUs at the 0 time point) * 100.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>47DEVDAEQ with caspase 3</th>
<th>47DEVDAEQ without caspase 3</th>
<th>24DEVDAEQ with caspase 3</th>
<th>24DEVDAEQ without caspase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>88.55</td>
<td>104.93</td>
<td>107.57</td>
<td>94.73</td>
</tr>
<tr>
<td>1</td>
<td>90.40</td>
<td>105.79</td>
<td>100.63</td>
<td>80.49</td>
</tr>
<tr>
<td>2</td>
<td>87.26</td>
<td>104.37</td>
<td>85.09</td>
<td>81.82</td>
</tr>
<tr>
<td>4</td>
<td>89.50</td>
<td>96.99</td>
<td>68.35</td>
<td>69.22</td>
</tr>
<tr>
<td>5</td>
<td>77.61</td>
<td>95.37</td>
<td>62.40</td>
<td>61.57</td>
</tr>
<tr>
<td>6</td>
<td>80.10</td>
<td>91.63</td>
<td>56.56</td>
<td>54.75</td>
</tr>
<tr>
<td>7</td>
<td>81.82</td>
<td>93.35</td>
<td>44.94</td>
<td>64.19</td>
</tr>
<tr>
<td>8</td>
<td>73.42</td>
<td>79.14</td>
<td>39.38</td>
<td>43.19</td>
</tr>
<tr>
<td>24</td>
<td>33.12</td>
<td>27.59</td>
<td>7.29</td>
<td>8.32</td>
</tr>
<tr>
<td>27</td>
<td>28.18</td>
<td>27.58</td>
<td>5.39</td>
<td>6.84</td>
</tr>
<tr>
<td>30</td>
<td>21.57</td>
<td>20.15</td>
<td>3.51</td>
<td>4.06</td>
</tr>
<tr>
<td>32</td>
<td>17.27</td>
<td>16.84</td>
<td>0.38</td>
<td>0.32</td>
</tr>
<tr>
<td>96</td>
<td>0.04</td>
<td>0.03</td>
<td>0.32</td>
<td>0.20</td>
</tr>
</tbody>
</table>
Figure 5.3. Western Blots. a.) 47DEVDAEQ substrate. b.) 24DEVDAEQ substrate. For both: lane 1 is uncleaved substrate, lane 2 is Benchmark Prestained ladder (Invitrogen), lane 3 is flow through fraction and the remaining lanes are elution fractions 1-4. The ladder bands correspond from top (pink band) to bottom to 60.4 kDa, 47.2 kDa, 35.1 kDa, 24.9 kDa, 18.3 kDa, 13.7 kDa and 5.7 kDa.
crystal structure of AEQ (Figure 5.1) revealed that the EF-hand I, consisting of amino acids 13-43, has an aspartic acid (D) as amino acid 24, which is located within the outerloop between the two helixes where calcium binds. Since the D was already present we replaced amino acids 25-27 (VNH) with EVD, so that the DEVD sequence would be created within the calcium binding loop. For the resulting protein, 24DEVDAEQ, we hypothesized that caspase-3 will cleave at the DEVD site, thus disrupting the EF-hand I and preventing the binding of calcium, which should lead to a significant loss in bioluminescence.

The 24DEVDAEQ protein was expressed and purified in the same manner as the 47DEVDAEQ protein. The purified 24DEVDAEQ was found to have a specific activity of $1.51 \times 10^9$ RLU/mg, which was similar to that of 47DEVDAEQ and sufficiently high for the protein to be employed as a substrate for caspase-3. The possibility of using the 24DEVDAEQ as a protein switch sensing system was evaluated in experiments identical to those set up for 47DEVDAEQ. The results are presented in Table 5.1. These results show a similar outcome as for the 47DEVDAEQ, where the bioluminescence activity, both in the presence and absence of caspase-3, decreased in a similar manner. As performed with the 47DEVDAEQ, the caspase-3 / 24DEVDAEQ reaction mixture (after 96 h incubation) was subjected to affinity chromatography using a Ni-NTA agarose resin column, and the cleavage pattern of the enzyme was analyzed by western blot. The 24DEVDAEQ, after successful cleavage by the caspase-3 enzyme, should generate two fragments that produce bands at 5,427 and 18,484 Da, corresponding to the N- and C-terminus of the aequorin, respectively. The western blot analysis (Figure 5.3b) shows a band at approximately 18 kDa in the flow through fraction, but no band around the 5,427 Da region is observed in the elution fractions, possibly due to the same reasons suggested for not seeing the 7,982 Da band from the 47DEVDAEQ substrate. Therefore, we believe that the 24DEVDAEQ substrate is also being cleaved by the caspase-3 enzyme but is still retaining its activity after cleavage.

For both reaction mixtures (caspase-3 / 47DEVDAEQ and caspase-3 / 24DEVDAEQ), the bioluminescence activity was measured in the flow through fraction
and the first elution fraction from the Ni-NTA columns (Table 5.2). The fact that the flow through fractions, containing aequorin amino acids 48-189 or 28-189, had some activity suggests that the EF-hand I can be partially or fully removed from the full length AEQ sequence and the resulting truncated proteins still yield bioluminescence. Furthermore, when each flow through fraction (C-terminal fragment) was combined with its respective first elution fraction (N-terminal fragment), an increase in the bioluminescence activity was observed (Table 5.2). This suggests that aequorin can be cleaved and recombined to regain some of its bioluminescence activity. Even though this data implies that the larger AEQ fragments can bioluminesce and can come together in solution with their smaller fragments, there is still the possibility that the obtained data could be distorted by incomplete cleavage of the aequorin substrates. Therefore, to verify our data, the truncated AEQs were expressed as individual fragments.

In view of the previous results, we hypothesize that the expression of functional truncated aequorin proteins is feasible. This shifted our short- and long-term goals toward discovering functional auto-bioluminescent AEQ fragments, as well as inactive AEQ fragments that are able to regain activity upon self reassembly or assisted reassembly. Moreover, understanding the mechanism by which aequorin emits bioluminescence is of considerable interest. It was shown previously that in order for aequorin to emit bioluminescence two of the three calcium binding EF-hands need to be occupied by calcium ions. More recently, it was also reported that EF-hand I has a lower binding affinity than EF-hands III and IV. Accordingly, the expression of truncated AEQs containing only one or two calcium binding sites may lead to functional truncated proteins, thus allowing to prove which of the EF-hands must bind calcium in order to produce bioluminescence and which calcium binding site(s) is/are not necessary for activity of aequorin. Determining the EF-hand(s) of the aequorin molecule that is/are responsible for triggering the most significant light emission could lead to a better understanding of the bioluminescence mechanism as well as assist in the rational design of functional truncated fragments of aequorin for new bioanalytical applications.
Table 5.2. Bioluminescence activity of 47DEVDAEQ and 24DEVDAEQ fragments after caspase-3 cleavage and nickel affinity chromatography separation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Flow Through (RLU’s)</th>
<th>Fraction 1 (RLU’s)</th>
<th>Flow Through + Fraction 1 (RLU’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>47DEVDAEQ</td>
<td>1,032</td>
<td>151</td>
<td>30,873</td>
</tr>
<tr>
<td>24DEVDAEQ</td>
<td>12,387</td>
<td>291</td>
<td>31,163</td>
</tr>
</tbody>
</table>
To begin our search for functional AEQ fragments we have focused on expressing truncated AEQ fragments containing only one or two calcium-binding EF-hands and at least one of the three tyrosine, tryptophan and histidine triads involved in the binding of coelenterazine.\(^5\) Considering the primary and the tertiary structures of aequorin, one can rationally design various truncated aequorins meeting the criteria mentioned above. Aequorin has 189 amino acid residues and contains three calcium binding helix-loop-helix (EF-hand) domains and one non-calcium binding EF-hand (EF-hand II) domain. The EF-hand I, EF-hand III, and EF-hand IV consist of amino acid residues 13-43, 106-136, and 142-172, respectively (Figure 5.1).\(^156\) Each of these domains contains a calcium binding site in the loop between the two helixes. EF-hand II, consisting of the amino acid residues 52-98, does not contain a calcium binding site. Besides the location of the EF-hands, one should also consider the nature of the residues that bind the chromophore. Coelenterazine is mainly stabilized by three triads each consisting of tyrosine, tryptophan and histidine, which are arranged in close proximity to each other in the folded protein. Triad I contains H16, W86 and Y82, triad II contains H58, W108 and Y132, and triad III contains H169, W173 and Y184.\(^5\) Taking these structural characteristics into account as a starting point, truncated proteins containing 1) EF-hand I, 2) EF-hands II, III and IV, 3) EF-hands I and II, 4) EF-hands III and IV, 5) EF-hands I, II, and III, and 6) EF-hand IV can be designed and expressed separately (Table 5.3). All six of these truncated proteins include at least one calcium binding site and one residue that stabilizes coelenterazine.

To date only one truncated fragment, AEQ48-189, has been fully characterized; therefore, its design and the related experimental results will be further discussed. The PCR was used to amplify the gene sequence encoding for the apoaequorin amino acids 48-189; this amplified gene was then ligated into the pET28a(+) expression vector, which contains a glutathione S-transferase purification tag (GST-Tag) and a histidine purification tag (His-Tag) upstream of the multiple cloning site (MCS), thus creating pAEQ48-189. SDS-PAGE analysis verified the expression of apoAEQ48-189 as a soluble protein, showing a band at 48.5 kDa (GST-Tag plus apoAEQ48-189). The GST-apoAEQ48-189 protein was isolated via Ni-affinity purification. The purified protein was dialyzed against recombinant enterokinase cleavage buffer and cleaved with enterokinase.
Table 5.3. Characteristics of the designed and expressed truncated AEQs: The present EF-hands, amino acid residues and triad amino acid residues involved in coelenterazine binding are indicated.

<table>
<thead>
<tr>
<th>Truncated AEQ</th>
<th>EF-hands</th>
<th>Residues</th>
<th>Triad Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>1-47</td>
<td>H16</td>
</tr>
<tr>
<td>2</td>
<td>II, III, IV</td>
<td>48-189</td>
<td>H58, Y82, W86, W108, Y132, H169, W173, Y184</td>
</tr>
<tr>
<td>3</td>
<td>I, II</td>
<td>1-74</td>
<td>H16, H58</td>
</tr>
<tr>
<td>4</td>
<td>III, IV</td>
<td>75-189</td>
<td>Y82, W86, W108, Y132, H169, W173, Y184</td>
</tr>
<tr>
<td>5</td>
<td>I, II, III</td>
<td>1-136</td>
<td>Y82, W86, W108, Y132</td>
</tr>
<tr>
<td>6</td>
<td>IV</td>
<td>137-189</td>
<td>H169, W173, Y184</td>
</tr>
</tbody>
</table>
to separate the GST-Tag from the apoAEQ48-189. The enterokinase was removed from the reaction mixture using a cleavage capture kit and then His-Tag-based purification was performed to separate the GST-tag from apoAEQ48-189. Given that the GST-Tag is attached to the His-Tag, the GST-Tag fragment should bind to the nickel resin, whereas the apoAEQ48-189 should not bind and remain in the flow through fraction. SDS-PAGE was run to verify the separation (Figure 5.4). From the gel it appears that the two were separated, as indicated by the presence of the GST-Tag in the elution fractions with a band at 31.1 kDa and apoAEQ48-189 in the flow through with a band at 17.4 kDa. In addition, the presence of apoAEQ48-189 in the flow through was proven by western blot analysis (Figure 5.4).

Upon initial purification the AEQ48-189 fragment did not show any bioluminescence activity. Even though the fragment was expressed as a soluble protein, it was still possible that the lack of activity was due to improper folding. In order to check this possibility, the fragment was denatured with urea and refolded by slowly removing the urea via dialysis, as explained in the Methods section. Once the protein was refolded in this manner, it was found to be active with a specific activity of $1.78 \times 10^8$ RLU/mg. To characterize AEQ48-189, the apoprotein was incubated with a variety of coelenterazine analogues and the emission spectrum maxima and half-lives were determined (Table 5.4). It was found that the emission spectrum of AEQ48-189 with native coelenterazine had a 40 nm shift to the red when compared to the full length AEQ. The red shift is thought to be from the removal of the N-terminus of AEQ, which can influence the binding interactions of coelenterazine within the hydrophobic pocket. More specifically, H16 is known to stabilize coelenterazine via hydrogen bonding, so the removal of H16 is thought to be the cause of the large red shift. This data concurs with data for the AEQ mutant Y82F that also exhibits a red shift in the emission peak, given that H16 and Y82 are both part of triad I and stabilize the same hydroxyl group on coelenterazine. Additionally, when AEQ48-189 was paired with other coelenterazine analogues, the emission maxima were all red-shifted compared to the same coelenterazines with the full length AEQ (Table 5.4). Interestingly, AEQ48-189 paired with coelenterazine i had an emission maximum of 523 nm. To the best of our knowledge
Figure 5.4. ApoAEQ48-189 Purification. Left – SDS-PAGE gel. Lane 1 Mark 12 protein ladder (Invitrogen); Lane 2 His-Tag purification flow through fraction. Right – Western blot. Lane 1 His-Tag purification flow through fraction; Lane 2 empty; Lane 3 Benchmark pre-stained ladder (Invitrogen); Lane 4 native AEQ control.
Table 5.4. AEQ48-189 Characterization. Compared to full length AEQ, the emission maxima are all red-shifted, but follow the same trend as with the full length AEQ. The half-life data follows to some extent similar trend as full length AEQ.

<table>
<thead>
<tr>
<th>Coelenterazine</th>
<th>$\lambda_{\text{Max}}$ (nm)</th>
<th>Half Life (s)</th>
<th>$\lambda_{\text{Max}}$ (nm)</th>
<th>Half Life (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>510</td>
<td>1.39</td>
<td>472</td>
<td>0.59</td>
</tr>
<tr>
<td>I</td>
<td>523</td>
<td>9.14</td>
<td>484</td>
<td>14.3</td>
</tr>
<tr>
<td>cp</td>
<td>491</td>
<td>0.43</td>
<td>454</td>
<td>0.17</td>
</tr>
<tr>
<td>f</td>
<td>518</td>
<td>0.52</td>
<td>480</td>
<td>0.59</td>
</tr>
<tr>
<td>fcp</td>
<td>498</td>
<td>0.17</td>
<td>463</td>
<td>0.37</td>
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<td>h</td>
<td>508</td>
<td>0.14</td>
<td>472</td>
<td>0.23</td>
</tr>
<tr>
<td>ip</td>
<td>486</td>
<td>0.31</td>
<td>454</td>
<td>0.67</td>
</tr>
<tr>
<td>hcp</td>
<td>489</td>
<td>0.92</td>
<td>454</td>
<td>0.13</td>
</tr>
</tbody>
</table>
this is the largest red shift which has been seen with any AEQ variant. The half-life data was different to that of full length AEQ albeit to a small degree, suggesting that the bioluminescence kinetics of AEQ48-189 is comparable to that of full length AEQ. The slight difference in half-life is contributed to the arrangement of coelenterazine in the hydrophobic pocket with EF-hand I absent opening up the N-terminus.

To further characterize the AEQ48-189 fragment, a far-UV circular dichroism spectrum was obtained (Figure 5.5). The spectrum demonstrates that AEQ48-189 has less $\alpha$-helical content than the native AEQ shown by the increase in mean residue ellipticity (MRE) between 205 and 230 nm. This is to be expected considering that amino acids 1-47 were removed from AEQ48-189; therefore, full length AEQ has two more $\alpha$-helixes than AEQ48-189. Nevertheless, AEQ48-189 represents a functional AEQ fragment given that it has auto-bioluminescence. This fragment could be employed for multiplex analysis using wavelength resolution detection as, when paired with coelenterazine i, it shows an emission maximum of 523 nm, which is 78 nm away from the emission maximum of 445 nm exhibited by the previously described AEQW86F mutant paired with coelenterazine hcp.\textsuperscript{236} Furthermore, this fragment is small, which is ideal for genetic tagging, and the red shift may be valuable for \textit{in vivo} imaging applications.

In conclusion, we presented the novel idea of employing genetic engineering to develop functional AEQ fragments. To the best of our knowledge this is the first time AEQ has been fragmented and characterized. To date, only one of the six proposed fragments has been characterized; however, the fact that it is active and has characteristics different from the full length aequorin opens up a whole new avenue for AEQ investigation and utilization. Once the proposed six truncated proteins are characterized, the knowledge gained could be used to design and prepare more truncated AEQ fragments. It may be possible, as shown by the 24DEVDAEQ caspase-3 data, to have functional truncated proteins that have been split in the loop between the helixes of the EF-hands instead of being split between EF-hands. For example, in the truncated AEQ fragments containing residues 25-189 or 1-152 the first or last helix, respectively, are removed, which opens up the N or C terminal end of the protein while leaving all of
Figure 5.5. Far-UV CD spectra. The spectra were collected with a JASCO J-810 spectrometer (JASCO Ltd. UK) with a 1 nm bandwidth and using a 0.1 cm pathlength cuvette. The wavelength scans were corrected for the blank and converted into mean residue ellipticity (MRE).
the calcium binding sites unmodified. Functional AEQ fragments can aid in explaining the mechanism behind the spectral tuning of the bioluminescence of aequorin as well as be useful in various bioanalytical applications. We envision discovering truncated AEQ fragments that can be used as reporters for multianalyte detection, genetic tagging, protein tagging, and protein-protein interactions. The sensitivity, small size and amenability to rational tuning of truncated AEQs are expected to facilitate the development of original and superb bioanalytical systems.
CHAPTER SIX
CONCLUSIONS AND FUTURE PERSPECTIVES

Advancements in biotechnology have allowed for a rapid expansion of the applications of bioluminescent proteins in a number of fields, including clinical diagnostics and environmental monitoring. The greatest advantages of bioluminescent labels are the sensitivity of detection afforded in physiological samples due to the lack of background bioluminescence, the simplicity and cost-effectiveness of the instrumentation employed to measure bioluminescence, given that there is no need for an excitation source, and the non-hazardous nature of the labels. For these reasons, bioluminescent proteins, such as the calcium-regulated photoprotein, aequorin, are preferred over conventional fluorescent reporters when detecting in physiological and environmental samples. A current limitation of the photoprotein, aequorin, is the lack of diversity in the ways it can be employed as a reporter. Traditionally, full-length aequorin has served primarily as a reporter for calcium sensing, immunoassays and DNA hybridization assays. To increase the diversity of aequorin as a reporter, this dissertation described how we have expanded the employment of aequorin by developing aequorin-based protein switches and bioluminescence inhibition assays, as well as discovered truncated variants that could be used in various novel bioanalytical applications.

Chapter Two explains the development of the first aequorin-based protein switch sensing system, which resulted in a functional sensing system for glucose. The three-dimensional crystal structure of aequorin was evaluated to rationally design an insertion site for the glucose-binding protein (GBP), a hinge-motion binding protein. GBP was inserted in between amino acid residues 47 and 48 of the cysteine-free mutant of apoaequorin. The resultant purified fusion protein was found to respond to glucose in a dose-dependent manner. Specifically, the protein switch was shown to selectively detect glucose down to $1.0 \times 10^{-7}$ M. While this study demonstrated for the first time the development of an aequorin-based protein switch, more studies need to be conducted to improve the biosensing system’s analytical performance and applicability. One major enhancement to this protein switch is to tune the working range of the sensing system.
The physiological levels of glucose in human blood are from 2 to 20 mM, which are in the high end of the working range of our sensor. While the sensor in its current form is useful to detect hypoglycemic levels, tuning our protein switch to respond to higher levels of glucose would be optimal to monitor hyperglycemia. To achieve this, a variant of GBP that is less responsive to glucose could be inserted into aequorin at the same insertion site allowing for the quantitative detection of higher levels of glucose. Another possibility is to insert GBP into a different rationally designed site within aequorin, which could yield a protein switch with a less sensitive response. For example, GBP could be inserted in between EF-hands II and III or in between EF-hands III and IV. As discussed in Chapter Two, these possibilities should allow for aequorin to still be functional even though a protein has been inserted within its sequence. In some cases, rational design does not lead to the best performing protein switch or even to a functional protein switch; therefore, a randomly designed protein switch comprised of aequorin and GBP may result in a more advantageous sensing system for glucose. To create a randomly designed protein switch the method of circular permutation can be used. Another shortcoming of the developed protein switch is the length of time required to detect glucose. In the current assay setup, coelenterazine and glucose were added to apoaequorin-GBP at the same time and incubated overnight. To address this issue, time studies need to be performed and the order in which the two reagents are added to the fusion protein investigated.

Future work includes employing the protein switch for glucose in serum samples to determine the feasibility of using our aequorin-GBP switch in vivo or ex vivo. To that end, human serum samples will be spiked with glucose and incubated with the aequorin-GBP switch to obtain a dose-response curve. EDTA will be added to the samples to chelate the calcium ions that are naturally present in serum, in order to prevent premature triggering of aequorin bioluminescence. In addition, the stability of the protein switch would need to be investigated for a commercialized glucose sensor. If thermal stability is unsatisfactory, possible future work includes inserting a thermostable GBP from the thermophyle *Thermus thermophilus* into aequorin. Additionally, a more thermostable mutant of aequorin could be employed, such as the quadruple mutant,
S32T/E156V/Q168R/L170I, which was recently developed in our laboratory. Lastly, the miniaturization and incorporation of this assay into a “Lab-on-a-CD or “Lab-on-a-Chip” device should be explored to facilitate on-site applications.

Chapter Three details the design and preparation of an aequorin-sulfate-binding protein (SBP) protein switch. The rational design described in Chapter Two was followed for inserting SBP into aequorin, leading to a functional protein switch sensing system for sulfate. The obtained protein switch was determined to be suitable for detecting sulfate and the feasibility of detecting sulfate in real-life samples was shown. Specifically, sulfate was detected in spiked serum, tap water and simulated urine in the millimolar range, which encompasses the average levels found in these samples. One shortcoming of some commercial sulfate sensors is the interference of other ions. We found that our aequorin-SBP protein switch responds to selenate; however, selenate is present in the environment and in the human body at concentrations several orders of magnitude lower than sulfate; additionally, the binding affinity of SBP for selenate is lower than that for sulfate. Therefore, selenate should not interfere with sulfate sensing when using our sensor. Another limitation of some sulfate sensing systems is the time required for readout. In that regard, our sensing system is capable of detecting sulfate in fifteen minutes.

Further work with our aequorin-SBP sulfate sensing system will involve its optimization toward implementation of a commercial system. For this, a thorough stability study is required to determine the shelf-life as well as the thermostability of the sensor. The functionality of the protein switch will be evaluated at different temperatures and incubation times. If the protein turns out to be unstable, protein engineering, as discussed above, will be employed to develop a more stable aequorin-SBP switch sensing system. Once the stability of the sensing system is optimized, the method will be validated against a traditional method of detecting sulfate, such as the turbidimetric method. Future work could also be extended to testing clinical samples such as blood or urine from normal subjects and autistic patients, which have been found to have reduced levels of sulfate in their blood and elevated levels of sulfate in their urine. In addition,
environmental samples from water treatment plants or local bodies of water could be tested. Similarly to the aequorin-GBP protein switch for glucose sensing, our aequorin-SBP sensing system should be miniaturized and incorporated into portable devices for on-site detection of sulfate.

The next chapter in this dissertation describes the development of an aequorin-based screening system for hydroxylated polychlorinated biphenyls (OH-PCBs). This work began with the intention of creating an aequorin-based protein switch for the detection of OH-PCBs. We planned to insert the Hbp-R recognition/regulatory protein into aequorin such that when the OH-PCBs bind to Hbp-R the conformation change in this protein would propagate to aequorin causing a change in the bioluminescence signal. Upon expressing and purifying the fusion protein and evaluating the possibility of employing it as a sensing system, it was found, from a control experiment, that the OH-PCBs decrease the bioluminescence of aequorin alone. This observation showed that using the aequorin-Hbp-R as a sensing system for OH-PCBs is unfeasible. However, the reality that the OH-PCBs decreased the bioluminescence of aequorin is very intriguing; therefore, in Chapter Four we presented the development of an aequorin-based bioluminescence inhibition assay for the screening of various OH-PCBs. This system was tested with fifteen different coplanar or non-coplanar OH-PCBs ranging from one to five chlorine substituents and with hydroxyl groups at the ortho, meta or para positions. The majority of the examined OH-PCBs induced a dose-dependent decrease of the bioluminescence of aequorin and exhibited limits of detection in the micromolar to sub-micromolar range. The feasibility of detecting OH-PCBs in human serum and tap water was also shown. Additionally, selectivity studies were performed demonstrating that hydrophilic compounds and slightly hydrophobic compounds do not affect the bioluminescence of aequorin. However, four of the hydrophobic compounds tested, which are similar in structure to OH-PCBs, caused a decrease in bioluminescence. Given that these compounds are also environmental pollutants, this assay could be employed as a screening system for various organic pollutants.
Future work to enhance the analytical characteristics of this OH-PCB sensing system should focus on protein engineering to lower the detection limits and increase the selectivity of our system. OH-PCBs are present in many environments over broad concentration ranges; therefore, in some instances systems with lower detection limits are necessary. Several molecular biology techniques are used to alter the bioluminescence intensity, emission wavelength and half-life of aequorin. These characteristics are modified by changing the amino acid sequence of aequorin and/or employing synthetic coelenterazine analogues. Changes in the bioluminescence characteristics are explained by changes in the interactions that stabilize coelenterazine in the hydrophobic core of aequorin. Since we hypothesize that OH-PCBs are inhibiting bioluminescence in a non-competitive manner via binding in the hydrophobic pocket of aequorin, changing the interactions between coelenterazine and/or the OH-PCB with aequorin could lead to variations in the assay performance. It would be ideal to find a variant of aequorin that would bind to the OH-PCBs with higher affinity, which we hypothesize would give lower detection limits. Furthermore, finding a mutant that binds only to OH-PCBs, in addition to coelenterazine, would make the sensing system more selective; however, this could be very challenging if hydrophobic interactions are the main reason for OH-PCBs binding to aequorin. To rationally choose an aequorin mutant, which would bind selectively and/or more tightly to aequorin, further mechanistic studies are required. For instance, solving the crystal structure of aequorin bound to an OH-PCB and to an OH-PCB plus coelenterazine could provide a detailed description of the OH-PCB binding site and the interactions involved in the binding. Determining where the OH-PCB binds to aequorin allows for rationally designed mutants to enhance the interactions between aequorin and OH-PCBs. Another approach would be to investigate our current aequorin-based inhibition assay for OH-PCBs in the presence of synthetic coelenterazine analogues as opposed to native coelenterazine. Changing the coelenterazine may result in different interactions between aequorin and the OH-PCB leading to altered analytical parameters.

Chapter Five discusses our research in engineering truncated variants of aequorin. Originally, this work was intended for the development of an aequorin-based caspase-3 “on/off” switch system. The rationale was that inserting the caspase-3 recognition site
within the sequence of aequorin would allow for caspase-3 to cleave the site in the resultant protein, thus causing aequorin to split apart and therefore lose bioluminescence. However, when caspase-3 cleaved our fusion protein, bioluminescence was not lost. This finding prompted us to propose that aequorin could be truncated and maintain bioluminescence; alternatively, cleaved inactive aequorin fragments could be obtained that regain bioluminescence upon self-assembling or assisted assembling. To that end, we rationally designed six truncated aequorin mutants. So far, one truncated aequorin, 48-189, has been expressed, purified and characterized. Excitingly, it was determined that aequorin 48-189 was auto-bioluminescent and had a red shifted emission maximum of 510 nm with native coelenterazine, and 523 nm when paired with coelenterazine \( i \). Therefore, this truncated mutant can be employed in multi-analyte detection with other aequorin labels with different emission characteristics; one example is an aequorin mutant that is blue shifted, such as, the aequorin W86F mutant paired with coelenterazine \( hcp \). Moreover, this truncated aequorin (48-189) is the most red-shifted aequorin mutant discovered to date; therefore, it is more appropriate than native aequorin for \textit{in vivo} imaging due to better propagation of red light through animal tissue. Additionally, truncated mutants should be ideal for genetic tagging due to their smaller sizes.

The future directions of this line of research are seemingly endless. Besides characterizing the six proposed mutants, many other truncated aequorins could be considered. For instance, aequorin truncated mutants that are split within an EF-hand instead of between the EF-hands should be explored. Furthermore, the truncated aequorin mutants could be exposed to further modifications, such as, site-directed and random mutagenesis, chromophore alterations and non-natural amino acid incorporation to discover additional functional mutants. The future applications of truncated aequorin are countless and will rely on the functionality of each truncated fragment, depending on if they are auto-bioluminescent, they self-assemble to bioluminesce, or only assemble and emit light when fused or conjugated to protein interacting partners. For auto-bioluminescent fragments, the most exciting future direction is probably to employ a red-shifted mutant in \textit{in vivo} imaging or tagging. A current limitation of \textit{in vivo} genetic
tagging is the size of the reporter and, consequently, a small red-shifted aequorin mutant would be ideal for genetic tagging. A second future direction for auto-bioluminescent aequorin fragments is to employ them as reporters in multi-analyte detection. For self-assembled fragments, the fragments will be employed to tag and detect soluble or insoluble proteins in living cells. For instance, the self-assembling fragments will be fused to soluble or insoluble proteins and the ability of the self-assembling aequorin fragments to determine protein solubility in living cells will be evaluated. Non-self-assembling aequorin fragments would be studied as a new reporter in biomolecular complementation assays for monitoring protein-protein interactions. As a model system, one aequorin fragment could be fused to the messenger protein calmodulin, which our group has extensive experience with, and another fragment could be fused to the calmodulin binding peptide in order to assess truncated aequorins ability to monitor the interactions \textit{in vitro} and \textit{in vivo}. Regardless of the route chosen, protein engineering can be taken a step further to fine tune or stabilize the developed systems using standard molecular biology techniques.

In conclusion, we have exploited and combined numerous protein engineering ideas and molecular biology techniques to broaden the properties of aequorin and expand its bioanalytical applications. Previous to this dissertation work, aequorin was used as a reporter in one intact unit. Herein, we have demonstrated that the characteristics of aequorin can be modified by inserting sequences of interest within the aequorin sequence or by fragmenting the protein, thus potentially leading to a whole new array of original applications. We also showed an unprecedented way of employing full-length aequorin for sensing, based on the discovery of new interactions of aequorin with hydrophobic molecules. It is envisioned that research in this arena will provide investigators with novel diverse bioluminescence labels exhibiting better properties as compared to the existing fluorescence ones, which should enable a host of new bioanalytical and clinical methods. Specifically, the availability of a palette of bioluminescent labels should allow for the design and development of multiplex assays in clinical and environmental samples, the real-time imaging of more than one target compound in a specific location, as well as for the implementation of new gene probes and protein-protein interaction methods for use in genomics, proteomics and drug discovery.
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