Bioluminescence in the Photoprotein Aequorin

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Introduction

Figure 1. Aequorea aequorea.

Bioluminescence is the emission of visible light by a living organism. Bioluminescence can occur through many different photoprotein reactions. This study focuses on bioluminescence of the photoprotein aequorin. This protein is obtained from marine coelenterates, such as the jellyfish *Aequorea aequorea* (Fig. 1). Bioluminescent, marine organisms like these jellyfish use aequorin in a variety of different ways including mating, feeding and defense mechanisms (Meighen, 1991). Aequorin is a blue fluorescent protein that emits intense light at 465-495 nm and is a member of the calcium-regulated photoproteins (Vysotski et al., 2006). Aequorins are some of the best studied systems of bioluminescence and have been found to simply require the addition of Ca^{2+} to produce bioluminescent blue light (Deng, et al., 2005).

Discovery

The bioluminescent photoprotein, aequorin, was discovered in 1961 by Princeton University Research Biochemist, Dr. Osamu Shimomura. The aequorin protein was

named for the luminous jellyfish species, Aequorea *Aequorea*, in which it was first discovered. Dr. Shimomura had previous experience studying bioluminescence from his work on the ostracod, *Cypridina*. Using his past experience and a little creativity, Shimomura successfully isolated the aequorin molecule by 1962 (Shimomura 1995; Shimomura 2005; Vysotski and Lee 2004).

Initially, Dr. Shimomura extracted the aequorin from granular, light-emitting organs in jellyfish tissue, using a pH 4 buffer to reversibly inhibit the protein's luminescence. After extraction, luminescence was regained through reactivating the protein by neutralizing the solution with sodium bicarbonate. By placing an inactivated sample of aequorin in seawater, Shimomura discovered that the molecule's luminescence was activated by Ca^{2+} ions. This discovery enabled him to devise a simple extraction procedure for aequorin, using an EDTA buffer solution as an inhibitor of luminescence. Through this discovery, he was also able to demonstrate how aequorin's luminescence could be used to measure cellular Ca^{2+} concentrations (Shimomura 2005).

Dr. Shimomura continued his work on aequorin and in 1974, he and a team of fellow researchers were able to identify the aequorin chromophore, coelenterazine, and the energy transfer mechanism from aequorin to the Green Fluorescent Protein. The following year, in 1975, Shimomura characterized the regeneration of aequorin to its active form from apoaequorin. It was not until 1987 that the first semi-synthetic aequorins with different calcium ion sensitivities were synthesized and in 2000, aequorin's structure was characterized using X-ray crystallography (Head *et al*. 2000; Shimomura 2005.

Aequorin Structure

Quaternary Structure of Aequorin

It is thought that the photoprotein, aequorin, is a functional monomer, although its quaternary structure appears as two molecules through X-ray crystallography (Figure 2). The two molecules are structurally very similar, exhibiting a few slight differences in the surface side chain orientations (Head *et al.* 2000).

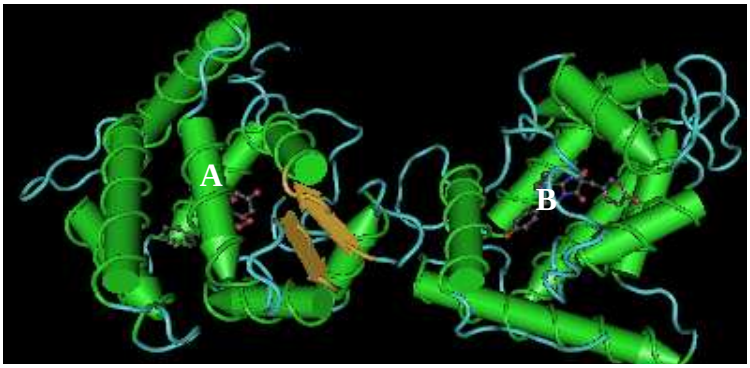

Figure 2. Crystal Structure of aequorin molecule shown as a pair of associated monomers A and B (Pubmed)

Aequorin Secondary Structure and EF-Hand Domains

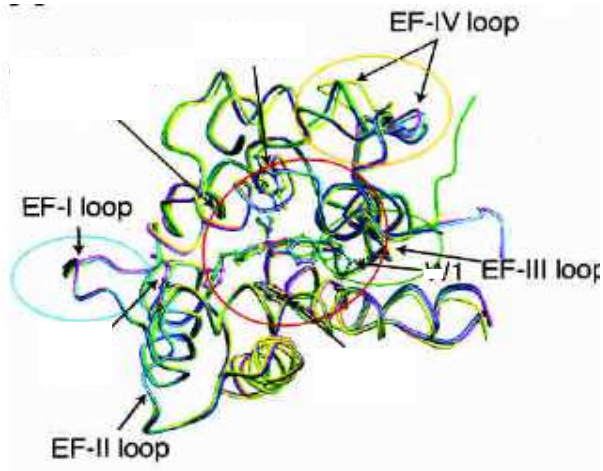

Figure 3. EF-Hand Domain pairs of Aequorin (Toma 2005)

Photoproteins, such as aequorin, have compact, globular, tertiary structures (Vysotski *et al*. 2006; Head *et al*. 2000). In aequorin, this globular structure is formed by the molecule's four EF-Hand domains, three of which are functional in binding calcium ions (Figure 3), (Head

et al. 2000; Vysotski and Lee 2004). Each EF-Hand domain is a helix-loop-helix motif, consisting of two left-handed α-helices that are joined by a tight, β-turn loop. The loop region of the EF-Hand domain contains twelve contiguous amino acid residues, from which the oxygen ligands, important for calcium ion binding, are derived (Deng *et al*. 2005). The four EF-Hands are arranged in pairs; with EF-I and EF-II forming a pair contained within the N-terminal domain, and EF-III and EF-IV forming the other pair, which is contained within the protein's C-terminal domain (Deng *et al*. 2005).

Also significant to aequorin's secondary structure are the unstructured regions visible near the N-terminus and the short stretches of anti-parallel β-sheets, formed by the back-to-back arrangement in each of the EF-Hand pairs (Head *et al*. 2000; Deng *et al*. 2005).

Aequorin's Hydrophobic Central Binding Cavity

Each symmetric half of the aequorin molecule is formed by the pairing of two EF-Hand domains in a cup-shaped arrangement. Situated within each cup, is a hydrophobic surface, lined with the side chains of hydrophobic amino acid residues. The two cup-shaped halves are brought together in a mouth-to-mouth conformation by the bend of a loop, which separates the second α-helix of EF-Hand II and the first α-helix of EF-Hand III (Head *et al*.

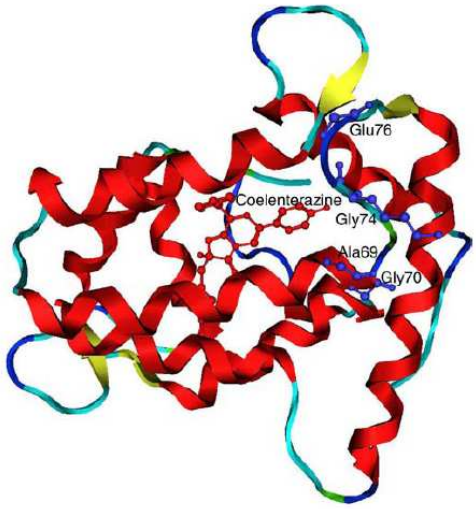

Figure 4. The hydrophobic central binding cavity containing the hydrophobic coelenterazine ligand (Head *et al.* 2000)

2000). This mouth-to-mouth alignment of the two halves forms the protein's hydrophobic central binding cavity. These two halves are held together through intramolecular hydrogen bonding between the EF-Hands. This bonding closes the central cavity and prevents access of the surrounding solvent into the cavity (Vysotski *et al*. 2006; Head *et al*. 2000).

The Kyte-Doolitle Hydropathic Index of aequorin indicates that the majority of the protein is comprised of hydrophilic domains (Figure 4). This describes the protein's hydrophilic external surface. Aequorin's inner cavity, however, contains hydrophobic amino acid residues from the α-helices in each of the four EF-Hand domains (Vysotski *et al*. 2006). The contributions of each EF-Hand domain to the hydrophobic central cavity appear as four distinct hydrophobic domains on the Kyte-Dootlittle Plot (Figure 5).

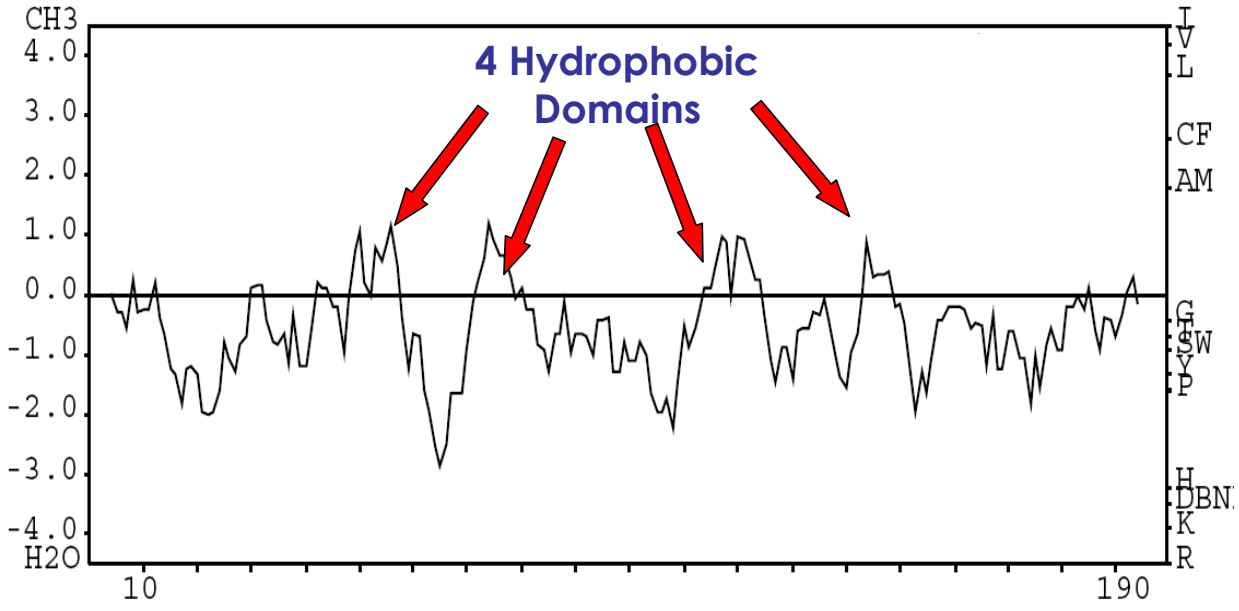

Figure 5. Kyte-Doolittle Hydropathic Index of Aequorin; Window = 7

Primary Structure and 2-Hydroperoxycoelenterazine Ligand

The photoprotein, Aequorin, is a single, polypeptide chain, consisting of 189 amino acid residues. This polypeptide chain weighs roughly twenty-two kDa (Vysotski and Lee 2005). The chain also contains the chromophoric ligand, 2-hydroperoxycoelenterazine, which is housed within the hydrophobic central cavity and is involved in the protein's bioluminescence reaction. All or part of twenty-one hydrophobic residues make up the lining of this central cavity. This includes three tyrosines, three histidines, two threonines, and one lysine (Head *et al*. 2000).

The 2-hyrdoperoxycoelenterazine substrate is tightly bound to the aequorin molecule through several weak interactions, including hydrophobic interactions and a series of hydrogen bonds. Most of the ligand's connection occurs in the EF-III Hand (Vysotski and Lee 2005). This connection is toward the C-terminus at Tyrosine 184 (Tyr 184) and Tyrosine 138 (Tyr 138) (Figure 9). Tyr 138 hydrogen bonds with N1 and Tyr 184 forms a hydrogen bond with the ligand's hydroperoxy group at C2. In addition to Tyr 184 and Tyr 138, there are three other hydrophilic residues within the hydrophobic domain. These residues are involved in the stabilization of the otherwise unstable 2-hydroperoxycoelenterazine ligand, through hydrogen bonding. These amino acids residues are Histidine 22 (His 22), Histidine 64 (His 64), and Histidine 175 (His 175) (Vysotski *et al*. 2006). Hydrogren bonding also anchors the C-terminal chain in its conformation and positions the side chain of Tyr 184 adjacent to Tyr 169 and the coelenterazine ligand (Head *et al*. 2000)

The 2-hydroperoxycoelenterazine ligand exists in a peroxidized form, consisting of a coelenterazine molecule coupled to a hydroperoxide. The peroxide group is attached

to the C2 position of coelenterazine in a chiral S configuration. The hydroperoxide appears to be stabilized by hydrogen bonding to the phenolic oxygen of Tyr 184. Coelenterazine is an imidizolopyrazinone ring formation (Head *et al*. 2000; Deng *et al*. 2005).

Function of Aequorin

Formation of Aequorin

The formation of the aequorin protein occurs when 2-hydroperoxycoelenterazine is present in the active site of apoaequorin (Inouye and Sasaki, 2007). Prior to entering the active site, the substrate exists as coelenterazine. Upon entering the active site, coelenterazine undergoes peroxidation (Fig. 7), using molecular oxygen and hydrogen, and forms 2-hydroperoxycoelenterazine (Vysotski et al., 2006).

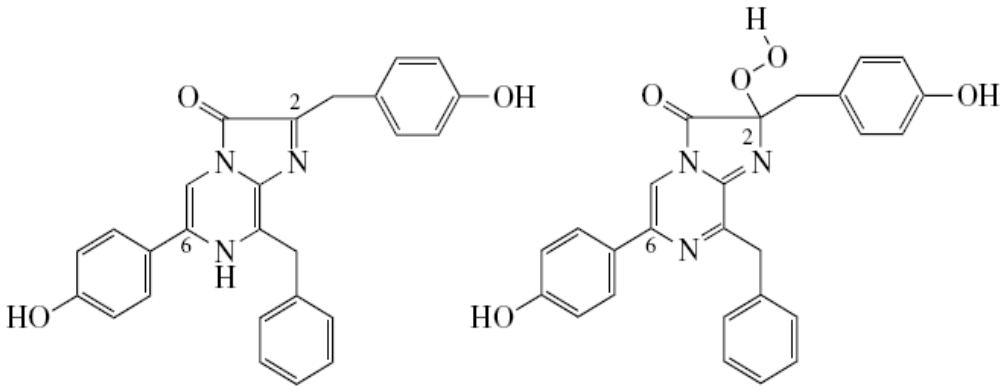

Figure 7. Chemical structures of coelenterazine prior to association with apoaequorin and 2-hydroperoxycoelenterazine after entering active site with highlighted peroxide group (Vyotski et al., 2006).

The active site in apoaequorin is highly hydrophobic with five key hydrophilic binding residues (His22, His64, His175, Tyr138 and Tyr184[1]) necessary for hydrogen-bond interactions with 2-hydroperoxycoelenterazine. These interactions allow for a tight, non-covalent bond between the two molecules (Deng et al., 2005). The peroxide group on the substrate is highly unstable but appears to be stabilized by its Hydrogen bond to Tyr184 (Vysotski and Lee, 2004). The stability of the peroxide is strengthened through the Hydrogen bond between Tyr184 and His175 residues (Inouye and Sasaki, 2007). This bond becomes important in later reactions involved in the bioluminescent process. Once 2-hydroperoxycoelenterazine is stabilized and interacting with residues in the active site of apoaequorin the two molecules form aequorin, and the protein complex is now responsive to calcium ions.

Ca^{2+} binding to EF-hand loops

The sites of Ca^{2+} binding in aequorin are the three active EF-hands (EFI, EFIII and EFIV) that have common helix-loop-helix structures consisting of two helices that attach to a homologous sequenced loop containing 12 adjacent residues. Of the 12 residues, 6 oxygen ligands are available for association with calcium ions resulting in the essential pentagonal bipyramidal array (Fig. 8). EF-hand II does not contain the residue sequence necessary for Ca^{2+} binding and is therefore not involved in the process of bioluminescence (Deng et al., 2005). The binding of Ca^{2+} to EF-hands is the first step in activation of bioluminescence. With the addition of calcium ions to the EF-hand binding

[1] Note: Positions of residues in sequence vary among papers by 6 spots. An example being that one source will refer to a residue as Tyr184 and another will refer to the same residue as Tyr190. We used the positions given in our most recent source of literature and used that position throughout the paper to maintain clarity.

sites results in molecule wide conformation changes. With just the slightest sign of calcium, aequorin is ready to react and bioluminescence is ready to happen.

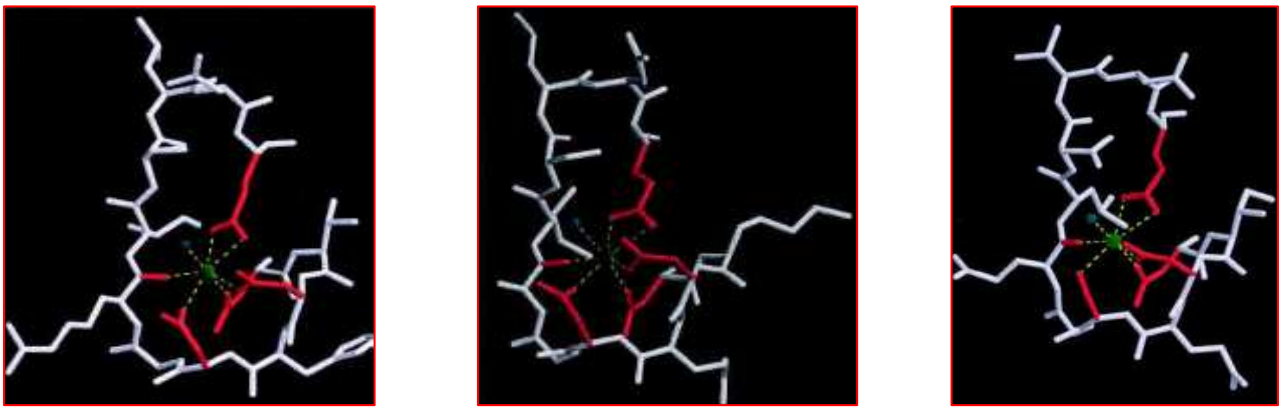

Figure 8. Three active EF-hands in pentagonal bipyramidal conformations when bound to Ca^{2+} (Deng et al., 2005).

Aequorin EF-hands are not equivalently structured to accept Ca^{2+}; therefore, they differ in their affinity for binding to calcium ions (Tricoire et al., 2006). EFI is the loop that is preformed to have to undergo less conformational changes in order to bind with calcium ions. Therefore, calcium ions will preferentially bind to EFI first (Deng et al., 2005). Thus, the first molecular event in bioluminescence involves the Ca^{2+} binding in EF-hand I and any conformational changes that result from the binding event. Upon binding to calcium and optimizing the pentagonal bipyramidal formation, EFI experiences a conformational twist that translates into the two α-helices attached to it (helix A and helix B; Fig. 9) (Vysotski and Lee,

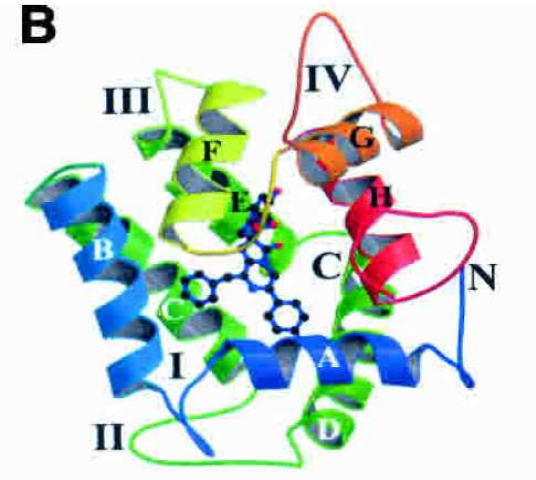

Figure 9. Aequorin diagram showing four calcium-binding EF-hands and their associated α-helices.

2004). α-Helix A is pulled toward the N-terminus during changes and increases in helical swirl. Since helix A is tightly bound with helix H, via lots of hydrogen bonds, the conformational change in A results in displacement of helix H (Deng et al., 2005). Therefore, the binding of one calcium ion in EFI results in the conformational change that occurs across the entire aequorin protein.

With conformational changes in Helix H occurring it is understandable that attached EF-hand IV undergoes conformational changes. Residues twist and hydrophilic residues change positions. These changes result in an increase in calcium binding in EFIV. Ca^{2+} binding occurs in the newly acclimated calcium-binding loop IV and attempts to optimize the pentagonal bipyramidal array of oxygen-calcium bonds (Vysotski and Lee, 2004). Again, helices directly attached to bonded loops experience the most change and α-helix H experiences a second shift in its conformation. This second change is the trigger for the reaction responsible for bioluminescence due to triggering of a decarboxylation reaction (Deng et al., 2005).

Since binding of just two calcium ions is necessary to initiate bioluminescence, conformational changes and Ca^{2+} binding in EF-hand on aid in increasing intensity of radiated blue light (Tricoire et al., 2006). After calcium binding in EFIV, the chain reaction of structural changes continues, despite the fact bioluminescence has already been initiated. Intrahelical changes in α-helix G, attached to EFIV, cause changes in helix F due to Hydrogen bond interactions between the helices. The changes in α-helix F translate to changes in EF-hand III and the affinity for calcium-binding increases. When Ca^{2+} binds with EFIII, resulting conformational changes aid in increasing the intensity of bioluminescent light (Deng et al., 2005).

Oxidative decarboxylation reaction:

Bioluminescence

The key step in the process of bioluminescence occurs when Ca^{2+} binding occurs in EF-hand IV and α-helix H is displaced for the second time (Deng et al., 2005). The change in helix H results in displacement of critical, 2-hydroperoxycoelenterazine-bound residues, His175 and Tyr184 (Inauye and Sasaki, 2007). With the movement of helix H the distance between His175 and Tyr184 decreases resulting in strengthening of the hydrogen bond and increasing the electrostatic interaction between the residues (Fig. 10). This can be considered a partial protonation of His175 by Tyr184 and in turn the hydroxide will protonate the Tyr184. The donor-acceptor separations around the 2-hydroperoxycoelenterazine trigger the destabilization of the substrate (Vysotski and Lee, 2004). The resulting peroxy anion will form a bond with C3 of 2-hydroperoxycoelenterazine and create an extremely unstable dioxyethane intermediate (Vysotski et al., 2006). Almost immediately, the coelenterazine experiences oxidative decarboxylation yielding an excited molecule of coelenteramide (Fig. 11) and carbon dioxide (Deng et al., 2005). When

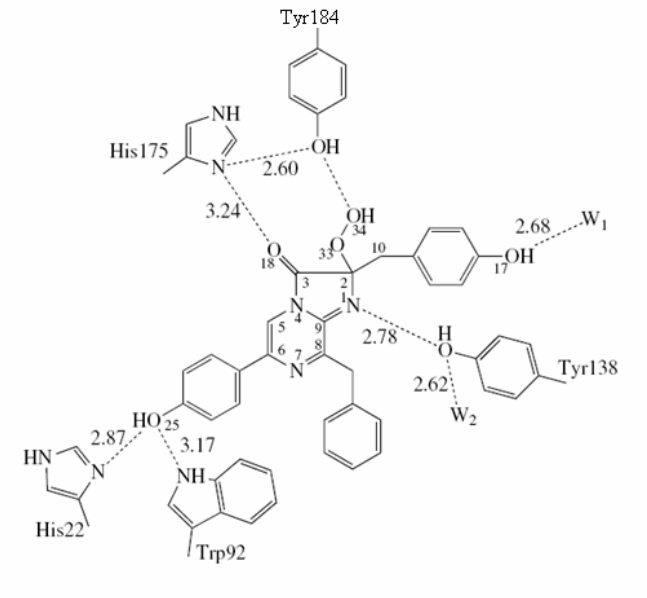

Figure 10. 2-hydroperoxycoelenterazine and its stabilizing bonds with His175 and Tyr184 of α-helix H (Vysotski et al., 2006).

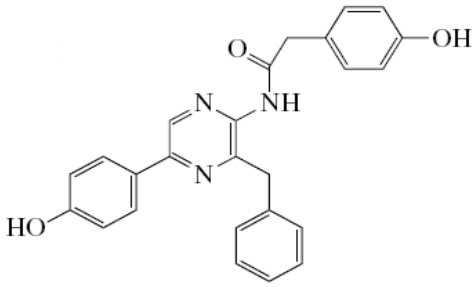

Figure 11. Coelenteramide molecule formed by decarboxylation of 2-hydroperoxycailenterazine.

coelenteramide returns from the excited state to the ground state the energy is released by the emission of light at 465-495 nm (Vysotski et al., 2006). Thus, the aequorin response to calcium occurs as a series of conformational changes ultimately being expressed as a flash of cool blue light (Fig. 12) that remains relatively constant throught the duration of calcium presence (Tricoire et al., 2006).

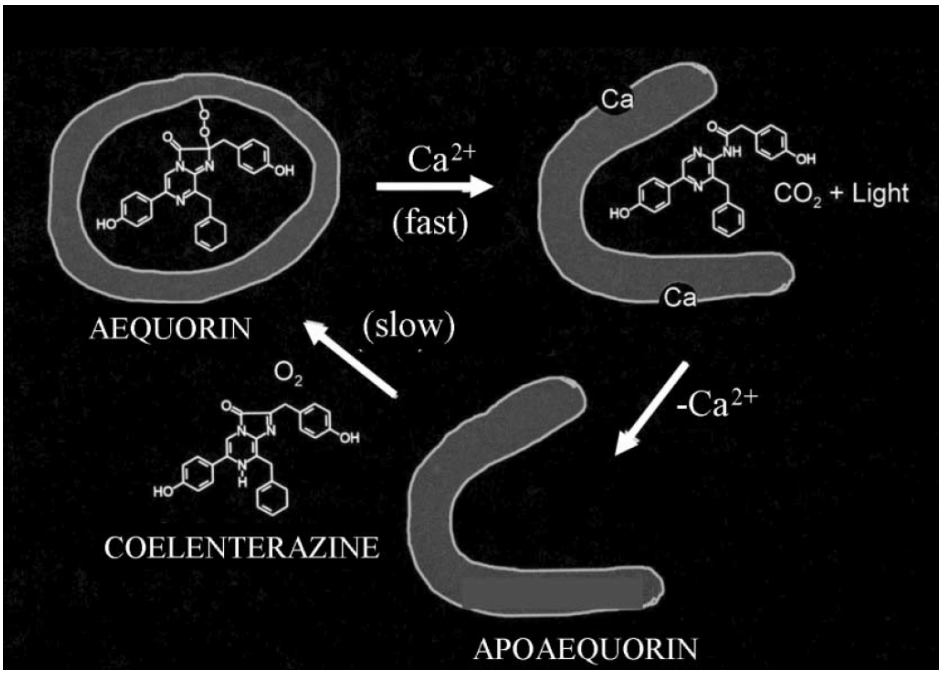

Figure 12. The overall reaction mechanism of bioluminescence. The reaction between apoaequorin and coelenterazine followed by reaction between aequorin and Ca^{2+} to produce coelenteramide, carbon dioxide and light (Shimomura, 2005).

Direction of Future Research

Bioluminescence continues to be of great interest amongst the scientific community. There is still much to be discovered about these beautiful and mysterious light-emitting reactions including the reaction mechanisms and the organisms that house

them. There is a driving force within many fields of experimental biology and medicine to utilize bioluminescent proteins in various analytical methods.

Since 1982, more than 1,000 scientific studies have been published in which aequorin was used. The use of aequorin as a Ca^{2+} indicator has transformed the field of cell physiology and allowed for some of the earliest work on Ca^{2+} signaling. In recent years, the rise of molecular biology has led to a renaissance in the use of aequorin. Scientists have now developed a method for expressing aequorin with target sequences. This allows the protein to be expressed only within specific organelles or locations within the cell. Localizing aequorin within a cell may aide in the understanding of local changes in calcium concentration which may be important for cell regulation (Vysotski *et al*. 2005). This technique has already allowed researchers to generate important new discoveries and will likely continue to do so in the future. Dr. Shimomura and his co-workers are continuing their study of aequorin's structure in an effort to better understand the mechanism by it emits light (Shimomura 2005).

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