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Cyclic AMP Receptor Protein-Aequorin Molecular Switch for Cyclic AMP

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Abstract

Molecular switches are designer molecules that combine the functionality of two individual proteins into one, capable of manifesting an “on/off” signal in response to a stimulus. These switches have unique properties and functionalities and thus, can be employed as nanosensors in a variety of applications. To that end, we have developed a bioluminescent molecular switch for cyclic AMP. Bioluminescence offers many advantages over fluorescence and other detection methods including the fact that there is essentially zero background signal in physiological fluids, allowing for more sensitive detection and monitoring. The switch was created by combining the properties of the cyclic AMP receptor protein (CRP), a transcriptional regulatory protein from E. coli that binds selectively to cAMP with those of aequorin, a bioluminescent photoprotein native of the jellyfish Aequorea victoria. Genetic manipulation to split the genetic coding sequence of aequorin in two and genetically attach the fragments to the N and C termini of CRP, resulted in a hybrid protein molecular switch. The conformational change experienced by CRP upon the binding of cyclic AMP is suspected to result in the observed loss of bioluminescent signal from aequorin. The “on/off” bioluminescence can be modulated by cyclic AMP over a range of several orders of magnitude in a linear fashion in addition to the capacity to detect changes in cellular cyclic AMP of intact cells exposed to different external stimuli without the need to lyse the cells. We envision that the molecular switch could find applications in vitro as well as in vivo cyclic AMP detection and/or imaging.

Introduction

Nature is able to produce proteins exhibiting highly desirable characteristics in terms of function, specificity, and efficiency, unmatched by synthetically developed molecules. Driven by this phenomenon, many scientists utilize and manipulate natural proteins to achieve the specificity and response that many of today's assays and detection methods strive for. But there is not always a single natural protein suitable for a task at hand. In some instances it would be advantageous to couple the functions of two completely unrelated proteins to work together as one to achieve a desired function-e.g., to quantify an analyte binding induced conformational change of a protein that naturally would be undetectable. Molecular switches can be the solution to accomplish such tasks. A protein molecular switch

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is defined as an entity that couples signal to functionality (1). For example, a protein molecular switch responds to an input signal by producing an “on/off” output signal. Such a protein could be constructed by genetically combining the coding sequence of one protein with that of another, creating a hybrid fusion protein with a completely new function, which displays the discrete functions of the individual proteins (1-4). To that end, we have inserted the flexible hinge motion binding protein, cyclic AMP (cAMP) receptor protein (CRP), into the genetic sequence of the bioluminescent protein aequorin (AEQ) to create a functional fusion hybrid protein, CRPAEQ. The separate proteins were genetically attached in a manner so that the conformational change experienced by CRP upon binding to cAMP results in a loss of bioluminescent signal of the AEQ portion of the hybrid protein (Figure 1).

AEQ (Figure 2) is a calcium dependent bioluminescent protein found naturally in the jellyfish *Aequorea victoria*. AEQ was originally isolated in 1962 by Shimomura *et al* (5, 6). The complete AEQ complex is composed of the apoprotein, apo-AEQ, and the organic chromophore coelenterazine. Apo-AEQ contains 189 amino acids arranged to form four EF-hand sequences (7, 8) that fold to create the hydrophobic core for the stabilization of the coelenterazine allowing for the bioluminescence to occur. In the presence of molecular oxygen and Ca^{2+}, AEQ undergoes a conformational change that allows for the oxidation of coelenterazine to coelenteramide with the release of CO_2 and the emission of light at 469 nm (9). Employing AEQ as a signal generating molecule has advantages over employing chemical or protein-based fluorescent probes because since the emission of light is the result of an internal biochemical reaction there is no need for an excitation source causing negligible background signal and leading to exceptional detection limits. In addition AEQ is environmentally friendly and thus it does not require special attention for disposal (10-12).

CRP regulates transcription of over 150 genes in *Escherichia coli* (13). It is allosterically activated by the binding of cAMP, which causes CRP to undergo a conformational change that allows for the binding of specific DNA sequences (14-16). In nature CRP exists as a homodimer of two identical subunits each containing 209 amino acids (23.6 kDa molecular mass) (16). Each subunit folds into two domains: the N-terminal domain (residues 1-133) responsible for the dimerization of CRP and housing the higher affinity cAMP binding pocket, and the C-terminal domain (residues 139-209), which is responsible for the interaction with DNA through a helix-turn-helix motif (13, 16, 17). Amino acids 134-138 form a flexible hinge that couples the two domains through covalent interactions (16). Each subunit contains two cAMP binding sites. The the N-terminal domain houses the higher affinity cAMP binding site, which binds cAMP in the μM range. The second cAMP binding site is located between the hinge and the turn of the helix-turn-helix motif and only binds cAMP when it is present at mM concentrations. Thus, the CRP protein is believed to exist in three states: free CRP with no cAMP bound, CRP-$(cAMP)_2$ when each subunit contains one bound cAMP molecule in the higher affinity binding site, and CRP-$(cAMP)_4$ when each subunit has two cAMP molecules bound, one each in the higher and lower affinity binding sites (13, 18, 19). The binding of cAMP by the higher affinity binding site is what induces the first conformational change in the N-terminal domain, which is then conveyed to the C-terminal domain resulting in a further conformational adjustment allowing the binding of DNA (13).

The large diversity of biological molecules exhibiting high specificity and affinity for analytes of interest has lead to the increasing use of these molecules for the development of novel biosensing systems. Molecular switches have been created to translate the binding induced conformational change of many of the protein biomolecular recognition elements into a quantifiable signal in the forms of altered fluorescence emissions, electron transfer, and biochemical activity among others. Examples of analytes targeted by such switches

include a number of different sugars, secondary messengers, drug molecules, and proteins to name a few (20). Molecular switches have also been developed based on DNA-base mispairing to monitor changes in the pH of the local environment (21). However very rarely has bioluminescence, with the advantages mentioned previously, been employed for the evolution of a molecular switch. Examples can be seen with the molecular switches created for glucose using the bioluminescent proteins AEQ (3) and firefly luciferase (22), but are limited beyond these examples.

Herein, we have employed CRP and AEQ in the construction of a molecular switch hybrid protein, CRPAEQ capable of the detection of cAMP. In cells, cAMP is an omnipresent secondary messenger with a plethora of functions including the regulation of cellular events (23-27). The ability to monitor cellular cAMP should provide insight into many cellular processes and responses from external stimuli. CRP is a favorable candidate for use in a molecular switch given that its hinge region allows for the protein to move in a clamping-type of motion when binding to cAMP, allowing for the possibility of a molecule attached to the ends of the protein to experience a greater change in position when the motion caused by the conformational change occurs. In our “on/off” molecular switch, cAMP provides the input signal when it selectively binds to CRP and induces the conformational change in the native protein, while AEQ provides the output signal in the form of the bioluminescence emission. To prepare the hybrid protein, AEQ was split in two fragments and attached to the N and C terminal ends of CRP to take advantage of this characteristic. We had previously observed that that there is a flexible loop between the EF hands I and II of AEQ, corresponding to amino acids 46-51, that allow for insertion of another protein without significantly disrupting the rest of the tertiary structure of the protein (3). We, therefore, hypothesized that by inserting CRP between amino acids 47 and 48 in addition to adding a linker sequence to both ends of the CRP molecule the AEQ fragments would have enough flexibility and freedom to reunite and reassemble to form the active bioluminescent AEQ complex.

Experimental Procedures

Materials

Tris free base – Tris(hydroxymethyl)amino methane was purchased from Serva (Heidelburg, Germany). Disodium ethylenediaminetetraacetate (EDTA), Luria-Bertani (LB) Agar, and LB Broth were purchased from Fischer Scientific (Fair Lawn, NJ). Urea, glucose, albumin from bovine serum (BSA), calcium chloride, 3′-5′-cyclic adenosine monophosphate, 3′-5′-cyclic guanosine monophosphate, 3′-5′-cyclic inosine monophosphate, 3′-5′-cyclic uridine monophosphate, 3′-5′-cyclic cytosine monophosphate, ampicillin sodium salt, chloramphenicol, kanamycin monosulfate, and ethidium bromide were purchased from Sigma-Aldrich (St. Louis, MO). Sodium Dodecyl Sulfate (SDS) was purchased from Curtin Mathesin Scientific, Inc. (Houston, TX). Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Gold Biotechnology, Inc. (St. Louis, MO). DEAE Fast Flow was purchased from GE Life Sciences. Poros 50 HQ was purchased from AB Applied Biosystems (Foster City, CA). The Bradford protein assay kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Mini-prep kits and gel extraction kits were purchased from Qiagen (Valencia, CA). Top10 and Top10F′ chemical competent cells were purchase from Invitrogen (Carlsbad, CA). The plasmid, pET28(a)+, BL21(DE3)pLysS chemical competent cells, and BL21(DE3) cells were purchased from Novagen (Madison, WI). JM109(DE3) chemical competent cells were purchased from Promega (Madison, WI). Gel code blue stain was purchased from Pierce (Rockford, IL). T4 DNA ligase, alkaline phosphatase, and EcoRI, HindIII, and Nhel, restriction enzymes were purchased from Promega (Madison, WI). Pfu Ultra polymerase and Taq polymerase were purchase from Strategene (Ceder Creek, TX). All chemicals were reagent grade or better and all aqueous solutions were

prepared using 16 mΩ deionized distilled water produced by a Milli-Q water purification system (Millipore, Bedford, MA).

Apparatus

Polymerase chain reactions (PCR) were performed using an Eppendorf Mastercycler Personal (AG, Hamburg). DNA electrophoresis was performed using a FB105 Fischer Biotech Electrophorese Power Supply (Pittsburgh, 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). Fusion proteins were purified using a BioCad Sprint Perfusion Chromatography System (Perseptive Biosystems, Framingtion, MA). Purity of the fusion 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). Bioluminescence measurements were made on an Optocomp I test tube luminometer (MGM Instruments, Inc., Hamden, CT) using Fischer borosilicate 12×75mm disposable glass tubes (Pittsburg, PA). Emission spectra were taken on a custom made SpectroScan instrument from Sciencewares (Farmingham, MA). Circular dichroism measurements were performed on a Jasco J-810 circular dichroism system (Easton, MD).

Construction, Expression, and Purification of CRPAEQ fusion protein—The CRPAEQ gene was produced by an overlap PCR method as shown in Figure 3. More specifically the PCR was used to amplify the coding sequence for amino acids 1-47 of AEQ and to introduce an EcoRI restriction site on the 5′ end of the coding sequence along with a sequence coding for a linker region with the sequence SGGGGS followed by the restriction sites for PstI and SacI at the 3′ end. The coding sequence for amino acids 48-189 of AEQ was amplified separately introducing PstI and SacI restriction sites and the linker region at the 5′end and a HindIII restriction site 3′ end of the coding sequence. By utilizing the AEQ 1-47 forward primer and the AEQ 48-189 reverse primer in an overlap PCR reaction the complete AEQ gene was reassembled with the linker region followed by the PstI and SacI restriction sites followed by another linker region inserted between amino acids 47 and 48 of AEQ. The overlap AEQ product was ligated into the EcoRI and HindIII sites of pIN4 to yield pOLAEQ. The gene for CRP was then amplified with PstI and SacI restriction sites introduced on 5′ and 3′ ends of the gene, respectfully, and inserted into the PstI and SacI sites of the AEQ gene in pIN4 to yield pCRPAEQ. The resultant fusion protein gene encodes for the split AEQ protein attached to the ends of CRP as follows: AEQ1-47-linker-CRP-linker-AEQ 48-189. After unsuccessful expression attempts were performed it was decided to remove the CRPAEQ fusion protein gene from the pIN4 plasmid and insert it into the pET28(a)+ plasmid. The PCR was used to introduce a NheI restriction site at the 5′end of the coding sequence while conserving the HindIII restriction site on the 3′end. The CRPAEQ gene was then ligated into pET28(a)+ to produce pCRPAEQ2. The DNA was then transformed into competent BL21(DE3) cells. Sequencing to confirm the correct DNA orientation was performed by the University of Kentucky Advanced Genetics Technology Center (AGTC).

Overnight cultures of the bacteria cells were grown at 37°C at 250 rpm in 5 mL LB broth containing 30 μg/mL Kanamycin. This culture was used to inoculate 500 mL of LB broth containing 30 μg/mL Kanamycin and grown at 37°C at 250 rpm. Once the OD_{600} reached 0.4 the cells were induced with IPTG to a concentration of 1 mM and the bacteria were allowed to grow overnight. The cells were collected by centrifugation at 12,000 rpm, 4°C for 15 minutes.

The CRPAEQ protein was only found to be expressed in inclusion bodies under a number of different conditions and thus required to be purified from the inclusion bodies. The cell pellet was dissolved in 20 mL of 30 mM Tris/HCl pH 7.5 2 mM EDTA buffer and lysed by two, 5 minute cycles of sonication alternating 10 s on/10 s off. The lyses mixture was then centrifuged for 10 min at 12,000 rpm and 4°C to collect the inclusion bodies. The pellet was then washed consecutively with 30 mM Tris/HCl pH 7.5, 2 mM EDTA, 150 mM NaCl; 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 1% Triton X-100; and then 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 5 mM $CaCl_2$ with the protein collected in between the washes by centrifugation at 12,000 rpm and 4° C for 10 minutes. The pellet was then re-suspended in 3 mL/g of pellet of 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 6 M Urea and allowed to rotate overnight at 4°C. The mixture was then centrifuged at 12,000 rpm and 4°C for 20 minutes and the supernatant, which contained the denatured CRPAEQ protein, was reserved. The denatured protein was purified using a DEAE Sepharose Fast Flow ion exchange column on a BioCAD Sprint Perfusion Chromatography System. The column was equilibrated with four column volumes of equilibration buffer (30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 6 M urea). The denatured protein supernatant was loaded onto the column and the column was washed with 3 column volumes of equilibration buffer. The protein was then eluted with elution buffer (30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 6 M urea, 1 M NaCl) using a 0 to 50% elution buffer gradient over 10 column volumes. Five mL fractions were collected and a SDS-PAGE was used to confirm the fractions containing the CRPAEQ protein, which were then pooled together. The urea was removed through dialysis in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA to allow the protein to refold. Protein refolding in the correct orientation was verified by bioluminescent activity of the AEQ portion of the protein. The protein concentration was determined to be 1.784 mg/mL using a Bradford protein assay.

Bioluminescent Half-life Determination—A final concentration of 1 μg/mL of coelenterazine was added to the apoAEQ-CRP fusion protein and left for 18 h at 4°C. Ten μl of the coelenterazine charged CRPAEQ mixture was added to a glass culture tube and the bioluminescence was measured using the Optocomp I test tube luminometer and the signal was collected for 10 s. The half life was determined using the one phase exponential decay kinetics equation from GraphPad Prism. The same procedure was followed for the half life determination of the CRPAEQ fusion protein in the presence of 0.1 mM cAMP.

cAMP Detection by CRPAEQ Fusion Protein—The CRPAEQ fusion protein was diluted to 1×10^{-5}M in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA and coelenterazine was added to a final concentration of 1 μg/ml. Ninety μl of the CRPAEQ/coelenterazine mixture was placed in a 1.5 ml polypropylene microcentrifuge tube and 10 μl of the corresponding cAMP standard was added to achieve the desired concentration and allowed to charge at 4°C for 18 h. Ten μl of the charged mixture was added to a disposable glass tube and the bioluminescence was measured using the Optocomp I luminometer as described above. The bioluminescent signal was integrated over six s.

Emission spectra—The CRPAEQ protein was charged with several native coelenterazine for 18 h at 4° C. The emission spectra of the protein with the native coelenterazine was then measured on the SpectroScan instrument from ScienceWares (Framingham, MA, USA).

Cellular cAMP Monitoring—Three separate BL21 (DE3) E. *coli* cell cultures were grown at 37° C in 8 mL LB broth at 250 rpm to an $O.D._{600}$ ~ 0.5. One culture was grown in the presence of 100 μM glucose, another in the presence of 50 mM inorganic phosphate, and the third without any additions. Once the $O.D._{600}$ of 0.5 was reached the cell cultures were distributed into 1 mL aliquots. Two μL of toluene was added to each aliquot and shaken at room temperature for 10 minutes at ~200 rpm. Fifty μL of the cell solution was then added

to 100 μL of a solution of CRPAEQ in 30 mM Tris/HCl, pH 7.5, 2 mM EDTA with 1 μL coelenterazine and allowed to charge 18 h at 4° C. Ten μL of each solution was then added to a disposable glass tube and the bioluminescent activity was observed using the Optocomp I as described above and integrating the signal over 6 s.

Results and Discussion

Bioluminescence has a number of advantages over fluorescence as a bioanalytical tool including extremely low background, highly sensitive detection and the ability to be used in the absence of an external excitation light source. Exhibiting these quality characteristics AEQ and has been shown to be a very sensitive label for a variety of applications (10-12). CRP is natively found in *Escherichia coli* and is used to regulate transcription of over 150 genes in the organism. CRP is allosterically activated by cAMP binding and upon binding cAMP undergoes a conformational change (15, 16, 29). This conformation change combined with the bioluminescent activity of AEQ can be integrated in the form of a fusion protein to create a very efficient and biologically relevant molecular switch to be implemented in a cAMP sensing system.

Much care, however, must be exhibited when genetically engineering the two proteins to be expressed together as a fusion protein. AEQ consists of four EF hands, three of which bind calcium (EF-hand I, III, and IV). It also has three triads consisting of the amino acids tryptophan, tyrosine, and histidine that are involved in the detainment of the chromophoric coelenterazine in the active site of the protein (3). These components are very important to the function of AEQ and must be conserved to preserve bioluminescent activity. Disrupting any of the structural components by inserting CRP would render the resulting protein bioluminescently inactive and thus useless for the designed function. However, there is a flexible loop on the exterior of the protein between EF-hand I and EF-hand II corresponding to amino acids 46-51 that has the ability to house a large sequence without disturbing the functional components of AEQ mentioned previously (3). Thus CRP was inserted between amino acids 47 and 48 of AEQ. A linker region was also included in between the AEQ and CRP fragments to allow for extra flexibility and aid in the correct folding of the individual portions of the protein. The gene encodes for the split AEQ protein sequence attached to the ends of the CRP sequence as follows: AEQ1-47-linker-CRP-linker-AEQ 48-189 (Figure 3). The fusion protein coding gene was then ligated into the pET28(a)+ expression plasmid. Following the transformation of the plasmid into BL21(DE3) cells an acceptable level of expression was achieved. The protein was expressed in inclusion bodies thus the inclusion bodies were solubilized through the use of urea and purified on a DEAE Sepharose ion exchange column also in the presence of urea. Refolding of the fusion protein was achieved by removing the urea through dialysis with 30 mM Tris/HCl, pH 7.5, 2 mM EDTA buffer. The purity of the protein was confirmed by a single prominent band at ~46 kDa on a SDS-PAGE gel, corresponding to the size of the CRP monomer added to that of AEQ. The protein also exhibited bioluminescent activity when charge with the coelenterazine and exposed to a Ca^{+} containing solution. The purified CRPAEQ was found to have a final concentration of 1.784 mg/ml as determined by a Bradford Assay.

The molecular switch showed a reproducible, decrease in signal corresponding to an increase in cAMP concentration from about 1×10^{-7} M to 1×10^{-3} M (Figure 4). It has been reported that CRP contains two cAMP binding sites, one higher affinity site located in the N-terminal domain and also a lower affinity site contained between the hinge region and turn of the helix-turn-helix motif of the protein (16, 19). The higher affinity cAMP binding site binds cAMP in the μM range, and the lower affinity site is bound when cAMP is in the mM range (13) keeping the observed response consistent with previous studies and permitting the sensor to be suitable for measuring cAMP levels of biologically relevant

samples (30). As reported above, as the cAMP concentration increases, the bioluminescent signal from the AEQ portion of the fusion protein decreases. It has been shown previously that CRP undergoes a hinge type conformational change upon the binding of cAMP (16). This leads to the presumption that upon binding of cAMP, the conformation change experienced by CRP occurs in a manner which causes the two halves of AEQ to move in opposite directions from each other based on their original position. With the spatial separation increased between the two halves, the bioluminescently active complex is unable to be reformed thus inhibiting the production of light upon the binding of Ca^{2+} by the AEQ portion of the protein as shown in Figure 1.

The spectral properties of the fusion protein were compared to that of the individual AEQ. The half-life of the bioluminescence from the AEQ of the CRPAEQ fusion protein was found to be 1.5 s without cAMP and 1.9 s with cAMP bound, similar to the 1 s half-life of the cysteine free mutant of AEQ (31). The emission maximum was slightly blue shifted to 480 nm, but still similar to the 471 nm emission shown by the cysteine free AEQ (31). This shows that although the CRP protein has been inserted in between the two halves of AEQ, the kinetics of the protein have not been greatly affected.

To test the switches potential in cAMP monitoring applications *Escherichia coli* was used as a model system. It has been shown previously in *Escherichia coli* that inorganic orthophosphate is able increase the amount of intracellular cAMP via two separate mechanisms. The inorganic orthophosphate not only stimulates adenylyl cyclase, the enzyme responsible for cAMP synthesis, but also slows down the degradation of cAMP by inhibiting cAMP phosphodiesterase, the enzyme responsible for the degradation, consequently elevating the cAMP levels in the cell (32). Glucose has been shown to possess a reverse effect on adenylyl cyclase, decreasing its activity, resulting in lower intracellular cAMP concentrations (33). Thus in an effort to allow for changes in cellular cAMP levels *Escherichia coli* cultures were grown in the presence of inorganic orthophosphate and glucose in hopes of increasing and decreasing cellular cAMP levels respectfully. Previous studies also indicate the use of toluene to increase cellular permeability is an effective and more efficient method to allow for the quantification of intracellular cAMP (32, 33). After allowing cultures to grow in the presence of the specified molecules, the CRPAEQ molecular switch was added to aliquots of the cell cultures and utilized to detect changes in cellular cAMP levels. As shown in Figure 5 the molecular switch provides a very reliable method to monitor increases and/or decreases in cellular levels of cAMP in response to different external stimuli without the need to lyse the cells. Although aequorin has traditionally been employed as a Ca^{2+} probe, in our experiments we employ EDTA buffered solutions to prevent uncontrolled bioluminescence emission triggered by endogenous Ca^{2+}. In addition, this ensures that the change in the bioluminescent signal is due solely to the changes in cAMP levels and not from Ca^{2+} fluctuations.

There are currently a variety of cAMP detection methods that have been developed and explored, including radiometric and antibody based assay systems (28). The radiometric systems pose the safety issues and disposal problems while the antibody based systems require expensive antibodies. Among those, we can cite the commercially available cAMP-Glo™ Assay from Promega (Promega Coorporation, Madison, WI, www.promeqa.com) and the LANCE® *Ultra* cAMP detection kit from PerkinElmer (PerkinElmer, Waltham, MA, www.perkinelmer.com). The cAMP-Glo™ assay uses a luciferase and protein kinase A to indirectly monitor the cAMP levels. cAMP binds to protein kinase A, releasing its catalytic subunits, which then catalyze the transfer of a terminal phosphate from ATP to the protein kinase A substrate, reducing the levels of ATP in the solution. The remaining ATP is then quantified with luciferase. Therefore, as the amount of cAMP increases, more ATP is consumed by the protein kinase A reaction, causing a decrease in the luminescence

observed. The LANCE® *Ultra* cAMP detection kit is a competitive binding assay between europium chelate labeled cAMP molecules and free cAMP in the sample. The two cAMP forms compete for binding sites on a fluorescently labeled anti-cAMP antibody. When the europium chelate molecule is excited, if it is bound to the antibody it transfers non-radatively energy to the attached fluorescent label, which, in turn, is excited and emits a distinct fluorescence signal. However, when the free cAMP is bound to the labeled antibody, there is no europium chelate capable of transfering energy to the fluorescent label, and therefore the fluorescence emission observed corresponds to the that of the europium on the labeled cAMP molecules. Thus, the intensity of the emission from the fluorescently labeled antibody is inversely proportional to the amount of free cAMP in the solution. Our CRPAEQ molecular switch offers a unique alternative to the available methods and creates a more direct detection method than those of the existing technologies. The ability of our switch to directly translate the binding of cAMP to a change in the bioluminescence intensity without requirement for the addition of reagents at different steps, results in a faster method of detection. Moreover, our switch is also cost-effective as the need for expensive labeled cAMP molecules and antibodies has been eliminated. This direct detection also reduces the possibility of the results being inaccurate due to potential changes in the concentration of the complementary assay molecules, such as ATP, that could be caused by unrelated cellular events or responses. In addition, bioluminescence offers the advantage of eliminating background interference from autofluorescent molecules commonly present in cellular and physiological samples. The CRPAEQ molecular switch provides a reagentless detection method saving resources while offering a safer and more environmentally friendly system. The hybrid protein which is the basis of the molecular switch, is expressed as a single protein. Thus, if the apoprotein is expressed and reconstituted *in vivo*, it could potentially find applications in cellular detection.

In summary, it has been demonstrated that the functions of two unrelated proteins, each with desirable characteristics can be successfully coupled in the development of a bioluminescent molecular switch which can be used for detection and monitoring of a specific analytes. CRP, a cAMP selective binding protein, was fused with a split AEQ, a photoprotein that generates a bioluminescent signal. When combined, the fusion hybrid protein results in a molecular switch capable of highly sensitive cAMP detection. This switch offers a number of advantages over currently employed methods in terms of simplicity, cost, safety and environmental friendliness. Moreover, it is envisioned that it would find a host of applications in a number of fields, such as biomedicine, biotechnology, medicinal chemistry, pharmacology, etc. Finally, CRPAEQ joins a very short list of bioluminescent molecular switches. Very rarely has a protein been able to be split, and re-united under desired conditions to give a reliable response able to be used for quantification of a specific analyte. This could open the doors to a whole new class of detection molecules to be implemented in various monitoring applications, allowing for a better understanding of mechanisms and behavior of complex biological and cellular systems as well as for elucidating the interactions and mechanisms of not-well characterized naturally occurring proteins.

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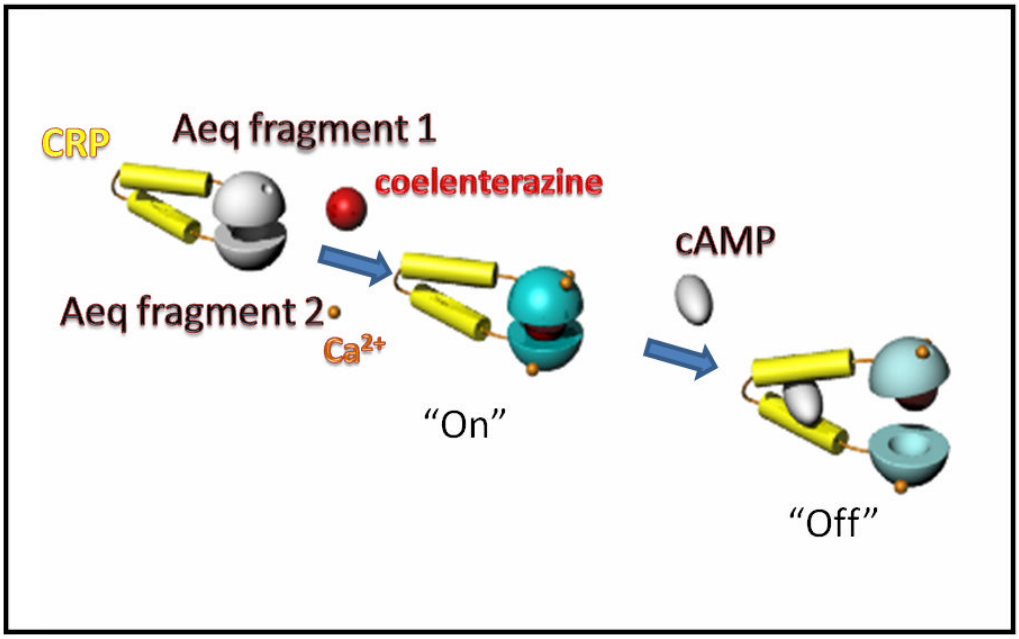

Figure 1.
Schematic of the response of the CRPAEQ molecular switch to the presence of cAMP. Initially the coelenterazine free CRPAEQ complex is not bioluminescent. Upon stabilization of the chromophoric coelenterazine and the addition of Ca^{2+} the complex will emit the bioluminescent flash, characteristic of normal AEQ. Following the cAMP induced conformational change the two halves of AEQ are positioned too far apart to form the active AEQ complex and thus decreases the observed bioluminescent intensity.

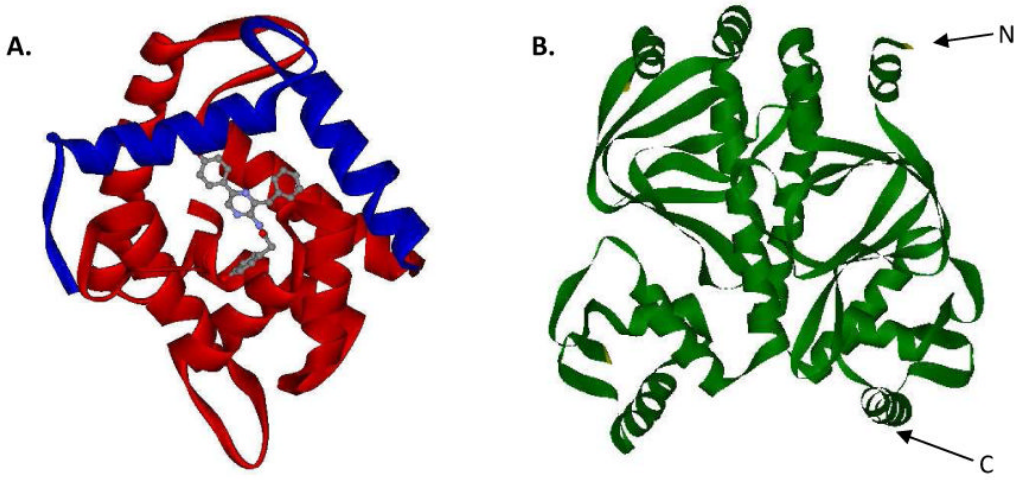

Figure 2.

A. The crystal structure of AEQ with coelenterazine bound in the hydrophobic pocket. Amino acids 1-47 are shown in blue and amino acids 48-189 in red representing the fragments in which AEQ was split. B. The crystal structure of CRP with the N and C termini labelled on one monomer of the homodimer.

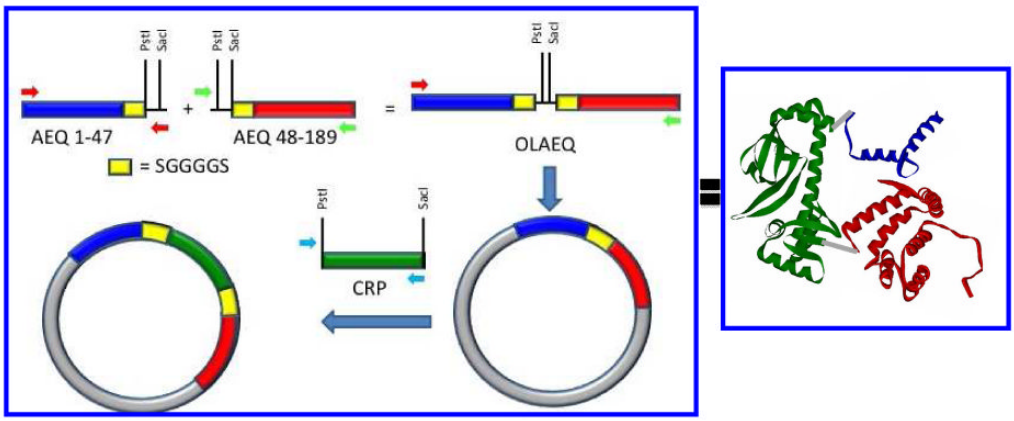

Figure 3.

Schematic of the stepwise design of the plasmid containing the gene for the CRPAEQ fusion protein. Briefly; the individual fragments of AEQ (amino acids 1-47 and 48-189) and CRP are PCR amplified while introducing unique restriction sites. Overlap PCR is then used to attach to two AEQ fragments followed by the ligation into the expression vector achieved through the cutting of unique restriction sites and ligated back together. The CRP gene is then inserted into the middle of the AEQ gene through the same restriction and ligation method. A schematic of how the fusion protein could potentially appear if crystalized is shown on the right.

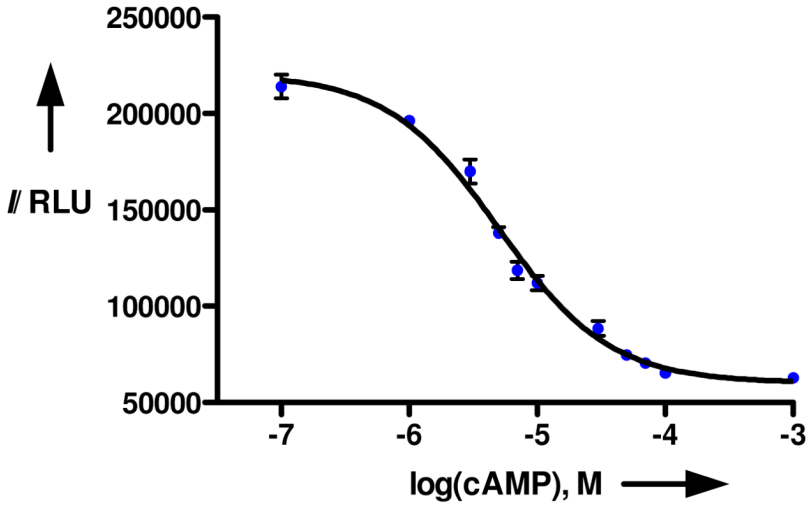

Figure 4.

Dose-response plot of the observed response of the CRPAEQ fusion protein to varying concentrations of cAMP. Data points are the mean of three samples in triplicate ± one standard deviation. Error bars not visible are concealed by the data point.

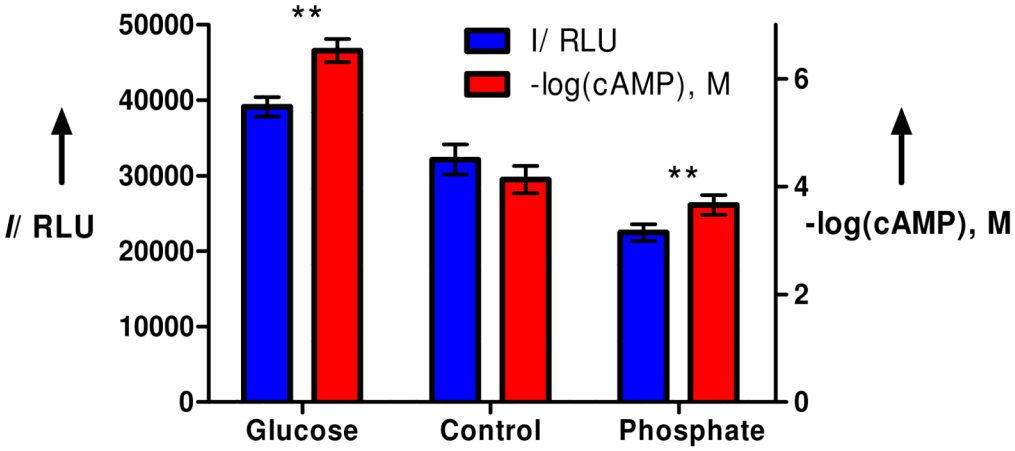

Figure 5.

The bioluminescent signal observed from the CRPAEQ molecular switch when exposed to cell cultures grown in the presence of either glucose, inorganic orthophosphate, or with no additions (Control). Concentrations were calculated from dose response plot of known concentrations. Points are mean of three samples in triplicate ± standard deviation. (** P value < 0.05 as determined via ANOVA compared to the Control culture).