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A PROTEIN SWITCH SENSING SYSTEM FOR THE QUANTIFICATION OF SULFATE

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Abstract

Protein engineering has generated versatile methods and technologies that have been instrumental in advancements in the fields of sensing, therapeutics and diagnostics. Herein, we demonstrate the employment of rational design to engineer a unique bioluminescence-based protein switch. A fusion protein switch combines two totally unrelated proteins, with distinct characteristics, in a manner such that the function of one protein is dependent on another. Herein we report a protein switch sensing system by insertion of the sulfate-binding protein (SBP) into the structure of the photoprotein aequorin (AEQ). In the presence of sulfate, SBP undergoes a conformational change bringing the two segments of AEQ together, “turning on” bioluminescence in a dose-dependent fashion, thus allowing quantitative detection of sulfate. A calibration plot was obtained by correlating the amount of bioluminescence generated with the concentration of sulfate present. The switch demonstrated selectivity and reproducibility, and a detection limit of 1.6×10^{-4} M for sulfate. Moreover, the sensing system was validated by performing sulfate detection in clinical and environmental samples, such as, serum, urine and tap water. The detection limits and working ranges in all three samples fall within the average normal / recommended sulfate levels in the respective matrices.

Keywords

Protein Switch; Protein Engineering; Binding Protein; Photoprotein; Sensing System

Introduction

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.[1,2] Protein switches incorporating optical detection have been employed to create sensing systems with tailored characteristics.[3,4,5,6,7,8] A protein switch consists of two unrelated proteins that

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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*. [9,10] It consists of the 189 amino acid apoprotein, apo-AEQ, and the organic chromophore coelenterazine (Fig. 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. [11] Three of the EF-hands have canonical Ca^{2+} -binding sequences, [12] while all four EF-hands fold together creating a hydrophobic core within the structure of the protein, which interacts and 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_2 with a concomitant emission of light at 470 nm. [13] 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 *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 [14] (Fig. 1(b)) that binds to tetrahedral, fully ionized, oxyacid dianions with high selectivity. [15] 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. [16,17] Moreover, the binding of sulfate causes a conformational change in the hinge region of the protein. [18] 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. [19] 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, [20,21] but elevated levels of sulfate in their urine. [22] The decrease in blood sulfate levels in autism patients is shown to be associated with impaired sulfation, [23] which can result in decreased detoxification. [24] 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. [25] 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. [26,27] 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; [28] 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 (Fig. 2). 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, 3.0×10^{-4} M [29] and 2.60×10^{-3} 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 (LaCl_3), 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 nitrotertrazolium 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 Matheson 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) GTGGCTAGCATGGTGAACTGACCAGCGACTTCGACAACCCAAGATG
G

- (2) GCCAGAGAGCTCGAGCTGCAGGCTACCACCACCACCCGTGTTATTGA
TGACA ATATCAG
- (3) CACAGGCTTTTAGGGGACAGCTCCACCGTAGAGCTTTTCGGAAGCAG
GATC
- (4) GTAGCCTGCAGCTCGAGCTCTCTGGCGGTGGCGGTTCTCTTGGAGCA
ACACCT GAGCAAG

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 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 48–189. 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 PCRs were performed using the Pfu 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 *Nhe*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 DNA Isolation Kit (Valencia, CA). The overlap apo-AEQ DNA was digested with *Nhe*I and *Hind*III restriction enzymes. The plasmid, pET-28a(+), was also digested with *Nhe*I and *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 *E. coli* BL21(DE3) cells, and the DNA was isolated.

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

- (5) GAGCTGCAGAAGGATATTCAGCTTCTTAACGT
- (6) CTTGAGCTCGCGTTTGCTGATCTGATCGAA

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 × 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 × 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 × 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 × 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 × 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 × 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 × 1.5 mL fractions of 10 mM Tris/HCl, pH 4.5, 100 mM NaH₂PO₄, 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 × 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 hybrid 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 hybrid apoprotein, and the apoprotein 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 μ L aliquots of 20 mg/mL 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) and 50 mg/mL nitrotertrazolium 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 16 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 μ L of SBP-AEQ hybrid protein with 50 μ L 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 μ L 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 1.0×10^{-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 μ g/mL and incubated at 4 °C for 16 hours. A 3.16×10^{-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 μ L of the hybrid protein. Sulfate-free control measurements were performed by mixing 20 μ L of 30 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.01% BSA buffer with 80 μ L of the hybrid protein. Each measurement was performed in triplicates. After 5, 10, 15, 30 and 60 minutes of incubation at room temperature, 5 μ L of the solutions was checked for bioluminescence activity by injecting 100 μ L 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 16 h. The emission maximum of the bioluminescence reaction was determined in the presence and absence of sulfate ions. For samples containing sulfate, 80 μ L of SBP-AEQ was added to three separate wells of a microtiter plate and 20 μ L of 3.16×10^{-3} M sodium sulfate was added. For control samples without sulfate, 80 μ L of SBP-AEQ was added to three separate wells of a microtiter plate and 20 μ L 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 μ L of triggering buffer. The bioluminescence signal was collected on a Spectro System Spectrographic Luminometer custom built by Scienceware, 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 16 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 μ L of SBP-AEQ was added to three separate wells of a microtiter plate and 20 μ L of 3.16×10^{-3} M sodium sulfate was added. For samples without sulfate, 80 μ L of SBP-AEQ was added to three separate wells of a microtiter plate and 20 μ L 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 μ L of triggering buffer, and bioluminescence emission intensity was measured on the FLUOstar Optima luminometer. The bioluminescence signal was collected for 6-s at 100 μ sec 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 $4000 \times g$ 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 1 μ g/mL. CD measurements were performed in the presence and absence of sulfate. For samples containing sulfate ions, 400 μ L of 3.16×10^{-3} M sodium sulfate, prepared in low salt buffer, was added to 1600 μ L of SBP-AEQ solution. For control samples without sulfate ions, 400 μ L of 5 mM phosphate, pH 7.0, 10 mM EDTA buffer was added to 1600 μ L 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 3.16×10^{-3} M sodium sulfate.

SBP-AEQ Response in Buffer

The stock solution of SBP-apo-AEQ hybrid protein was diluted to 1.0×10^{-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 μ g/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 μ L of the hybrid protein. For the control samples, 20 μ L of buffer was added to a microtiter plate wells containing 80 μ L 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 μ L 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 detection limits were determined by interpolating the average bioluminescence emission intensity 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, 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 1.0×10^{-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 μ g/mL. One hundred millimolar solutions of sodium selenate, sodium nitrate, sodium arsenate, potassium chloride, 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 μ L of each analyte was added to a microtiter plate well containing 80 μ L 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 μ L 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.[2,30,31] 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.[30] 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.[3] 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.[32] 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.[8,33]

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.[8] 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[18] 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 SGGGS 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* BL21 (DE3) 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 imidazole and adjust the pH for the subsequent ion exchange chromatography. Specifically, diethylaminoethyl (DEAE) sepharose resin was chosen as a strong 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 results of the purification demonstrated that the contaminating band at approximately 49 kDa was not retained by the anion exchange resin at the given pH conditions and was eluted from the column in the flow through, while the SBP-apo-AEQ hybrid protein was successfully purified. Then, the elution fractions containing the pure SBP-apo-AEQ protein were combined and the protein was renatured slowly via dialysis. Slow dialysis removes the urea from the denatured protein solution 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 1.56×10^9 RLU/mg and in the presence of sulfate was 1.97×10^9 RLU/mg. On the other hand, the parent aequorin had a specific activity of 3.65×10^{12} RLU/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 (Fig. 3). The near UV region is sensitive to small changes in the tertiary structure due to binding interactions or changes in the solvent.[34] The correct experimental controls were performed to assure that there was no change in the solvent during experiments. Therefore, a change in the molar ellipticity in the near UV region confirms that a change in the tertiary structure of the protein has occurred due to binding interactions. Figure 3 demonstrates a change in the near UV region indicated by the peak shift from 279 nm to 274 nm upon the addition of sulfate, 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 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 demonstrated that the least amount of SBP-AEQ protein that could be used was 1.0×10^{-7} M. Employing this concentration of hybrid protein, a time study to determine the optimal response time of the protein switch was performed. For this, SBP-AEQ was reacted with 3.16×10^{-3} M sulfate, and the bioluminescence emission was determined at various time periods. Incubation times shorter than 15 min generated a negligible change in bioluminescence 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 1.0×10^{-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 LaCl_3 , and the bioluminescence intensity was measured.

It should be mentioned that the bioluminescence of AEQ is generally triggered by Ca^{2+} ions yet other ions, such as Sr^{2+} , Cd^{2+} , Y^{3+} and La^{3+} , can also cause the emission of significant bioluminescence.[35] 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. Cd^{2+} 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 Y^{3+} or La^{3+} . [35] LaCl_3 was finally chosen as the triggering reagent given its simplicity of use and storage as compared to metal oxides such as Y_2O_3 . The calibration plot for sulfate (Fig. 4) 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 dose-response curve the detection limit for the sulfate ion is calculated to be 1.6×10^{-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 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 3.9×10^{-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 2.60×10^{-3} M[36]; 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 3.0×10^{-4} M.[29] 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.[22] The data in Table 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 had an average of 3.03×10^{-3} M sulfate in their urine, whereas a group of patients with autism had an average of 6.82×10^{-3} M sulfate excreted in their urine.[22] 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.

[15] 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 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 Fig. 5). 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 3.85×10^{-10} M [37] and serum selenium concentration is 9.65×10^{-7} M [38]. 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 [39]. 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 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 onsite 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 1.6×10^{-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.

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a.

b.

Fig. 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.

Fig. 2.
A schematic demonstrating the mechanism of the SBP-AEQ protein switch.

Fig. 3. 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 is represented by the solid line and SBP-AEQ in the absence of sulfate is represented by the dashed line. The addition of sulfate to SBP-AEQ induced a change in the tertiary structure of the protein.

Fig. 4. Dose-response curve obtained with the protein switch when exposed to various concentrations of sulfate in buffer. Specifically, sulfate solutions were incubated for 15 min with 1.0×10^{-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 $\pm$ one standard deviation.

Fig. 5. Selectivity studies employing the SBP-AEQ protein switch. Each anion was incubated for 15 min with 1.0×10^{-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 $\pm$ one standard deviation.

Table 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.

	Detection Limit (M)	Intra-assay RSD (%)	Working Range (M)	R ²
Buffer	1.6×10^{-4}	0.8 – 3.9	$1.6 \times 10^{-4} - 2.3 \times 10^{-3}$	0.93
Tap water	3.9×10^{-4}	0.9 – 4.9	$3.9 \times 10^{-4} - 4.7 \times 10^{-3}$	0.91
Serum	1.9×10^{-4}	0.7 – 4.6	$1.9 \times 10^{-4} - 2.3 \times 10^{-3}$	0.93
Urine	1.9×10^{-4}	1.0 – 4.3	$1.9 \times 10^{-4} - 3.5 \times 10^{-3}$	0.91