LETTER

ACS chemical biology

# Modulating the Bioluminescence Emission of Photoproteins by *in Vivo* Site-Directed Incorporation of Non-Natural Amino Acids

**Laura Rowe[†,§], Mark Ensor[†], Ryan Mehl[‡], and Sylvia Daunert[*,†]**

[†]Department of Chemistry, University of Kentucky, Rose Street, Lexington, Kentucky 40506 and [‡]Department of Chemistry, Franklin and Marshall College, 415 Harrisburg Avenue, Lancaster, Pennsylvania, 17603, [§]Current address: Department of Chemical Engineering and Biotechnology, University of Cambridge, Tennis Court Road, Cambridge, U.K., CB2 1TN

Light-emitting proteins have become valuable and versatile tools in a plethora of biological and chemical applications. The visualization capabilities of these light-emitting proteins has rendered them invaluable as imaging reagents in a variety of *in vitro*, *in vivo*, and *in situ* studies as well as being used as reporters in protein and cell assays, biosensors, environmental monitoring, biothreat assessment, and high-throughput screening of pharmaceuticals. Bioluminescent proteins in particular have received a significant amount of attention recently due to the advantages they possess over fluorescent proteins in terms of sensitivity of detection and due to their use in longitudinal and circadian studies (*1*). However, a current shortcoming of bioluminescent proteins lies in their limited spectral variety, and mutagenesis strategies aimed at rectifying this frequently cause partial or total loss of bioluminescent activity (*2*). In an effort to circumvent this challenge, we incorporated non-natural amino acids into an aromatic, hydrogen (H)-bonding residue position (Y82) within the chromophore-binding site of the bioluminescent photoprotein aequorin and combined the non-natural aequorins with altered chromophore analogues. Our strategy involved expanding the repertoire of amino acids available for translation into aequorin by employing a site-specific *in vivo* non-natural amino acid incorporation method. This resulted not only in the successful site-specific incorporation of non-natural amino acids *in vivo* into a bioluminescent protein for the first time but also in significant shifts of the bioluminescence emission maximum of aequorin. This study, therefore, presents a new method for rationally tuning bioluminescence color by producing new variants of aequorin using a format that will allow for the efficient 'scale-up' of these non-natural bioluminescent reporter proteins.

Aequorin is a globular, 22 kDa protein composed of four EF-hand binding domains. Aequorin contains a chromophore, coelenterazine, which is noncovalently bound to the interior hydrophobic core of the protein (Figure 1). When calcium (or several other alkali metals) binds to the EF-hand loops on the outer domains of aequorin, a conformational change occurs that leads to the oxidation of the bound coelenterazine to an excited coelenteramide, which relaxes *via* the emission of a bioluminescent photon ($\lambda_{em}$ = 473 nm) (*3*). Two general strategies have thus far been employed in altering the wavelength of emission of aequorin: site-directed natural mutagenesis and altering the structure of the coelenterazine chromophore (*4*–*6*). Previous work performed by us and others has shown that natural mutation of Tyr82 results in a shift of the emission maxima of aequorin while retaining high

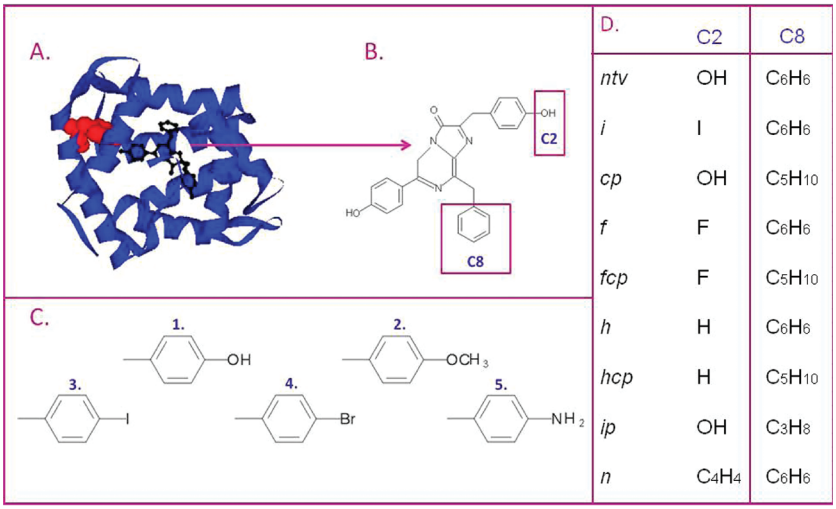
**ABSTRACT** The *in vivo* incorporation of non-natural amino acids into specific sites within proteins has become an extremely powerful tool for bio- and protein chemists in recent years. One avenue that has yet to be explored, however, is whether or not the incorporation of non-natural amino acids can tune the color of light emitted by bioluminescent proteins, whose light emission mechanisms are more complex and less well understood than their fluorescent counterparts. Bioluminescent proteins are becoming increasingly important in a variety of research fields, such as *in situ* imaging and the study of protein−protein interactions *in vivo*, and an increased spectral variety of bioluminescent reporters is needed for further progress. Thus, herein we report the first successful spectral shifting (44 nm) of a bioluminescent protein, aequorin, *via* the site-specific incorporation of several non-natural amino acids into an integral amino acid position within the aequorin structure *in vivo*.

*Corresponding author, daunert@uky.edu.

Received for review November 18, 2009 and accepted March 15, 2010.

Published online March 15, 2010

10.1021/cb9002909




| D. | C2 | C8 |
|---|---|---|
| *ntv* | OH | $C_6H_6$ |
| *i* | I | $C_6H_6$ |
| *cp* | OH | $C_5H_{10}$ |
| *f* | F | $C_6H_6$ |
| *fcp* | F | $C_5H_{10}$ |
| *h* | H | $C_6H_6$ |
| *hcp* | H | $C_5H_{10}$ |
| *ip* | OH | $C_3H_8$ |
| *n* | $C_4H_4$ | $C_6H_6$ |

**Figure 1. A) The X-ray crystal structure of aequorin with the tyrosine at position 82 (Tyr82) highlighted in red CPK and the coelenterazine chromophore in ball and stick representation. B) The coelenterazine analogues utilized had altered functionality at positions C2 and C8. C). The structures of the non-natural amino acids incorporated: the R group of the native Tyr residue (1), 4-methoxy-Phe (2), 4-iodo-Phe (3), 4-bromo-Phe (4), 4-amino-Phe (5). D) The structures of the C2 and C8 groups for the different coelenterazine analogues.**

bioluminescence activity. Since the natural Y82 to F82 mutation shifts the emission maxima of aequorin by approximately 30 nm, we postulated that the introduction of different non-natural amino acids into the Y82 position would also result in large shifts in the emission wavelength (*7*).

In order to site-specifically incorporate the non-natural amino acids into position 82, we utilized the amber codon, *in vivo* method using orthogonal tRNA and tRNA synthetases (see Methods) (*8, 9*). This non-natural amino acid incorporation method has been thoroughly developed and characterized by others, and each of these synthetases have previously been shown to incorporate a specific non-natural amino acid into solely a TAG codon efficiently and quantitatively (*10*). We chose four non-natural phenylalanine derivatives for incorporation: 4-bromo-F, 4-iodo-F, 4-amino-F, and 4-methoxy-F (Figure 1) due to their limited steric differences as compared to Tyr and to the altered electron-donating and H-bonding capacity of the functional groups.

In order to increase the diversity of bioluminescence properties, these four non-natural aequorin derivatives were also combined with coelenterazine analogues containing altered functionality at the C2 and C8 positions (see Figure 1). These have been previously shown to alter the emission maximum of aequorin while maintaining bioluminescence activity (*4, 5*). Therefore, all four of the non-natural and natural aequorin derivatives were charged overnight with 10 coelenterazine analogues, and the emission maximum and bioluminescence half-life of each non-natural aequorin−coelenterazine analogue pair was obtained (see Supplementary Tables 1 and 7).

Characterization of the newly prepared non-natural aequorin variants demonstrated that the incorporation of the non-natural amino acids resulted in a significant shift in the bioluminescence emission wavelength of aequorin. The maximal shift observed was when 4-methoxy-Phe was inserted at location 82 of aequorin (methoxy-82-Aeq). Specifically, methoxy-82-Aeq presented a 36 nm shift when paired with native coelenterazine and an impressive 44 nm shift when coelenterazine *i* was incorporated (Figure 2). The overall red shifts observed for the non-natural aequorin variants may be attributed to altering the H-bonding capacity and positioning of the protein with these mutations, since, as previously mentioned, the natural mutation from Tyr to Phe at position 82 yields a 20−30 nm red shift in cysteine-free and wild-type aequorin (*2, 7*). Furthermore, the data from Table 1 suggest that the incorporation of the non-natural amino acids 4-methoxy-F and 4-amino-F into aequorin affects all of the coelenterazine ligands in the same way, with the incorporation of 4-methoxy-F resulting in an approximately 35 nm bioluminescence red shift (31−37 nm) and 4-amino-F resulting in an approximately 12 nm bioluminescence red shift (10−14 nm) with all 10 of the coelenterazine analogues. However, the incorporation of 4-bromo-F, and to a lesser extent 4-iodo-F, resulted in different shifts depending on the coelenterazine analogue that the non-natural aequorin was paired with. For example, Bromo82AEQ gave a 20 nm red shift when paired with coelenterazine *native* and coelenterazine *i,* an 11−13 nm red shift with coelenterazines *cp*, *f*, and *fcp*, and merely a 4−8 nm red shift with coelenterazines *h*, *hcp*, *ip*, and *n*. The non-natural amino acids 4-amino-F and 4-methoxy-F can both contribute to the stabilizing H-bonding network that is known from structural studies to occur between aequorin (Y82, W86, and H16) and the phenolic group (non-C2 phenol) of coelenterazine, whereas 4-bromo-F and 4-iodo-F cannot. It is perhaps this fundamental difference that causes the H-bonding capable non-natural amino acids to give the same degree of red shift for every coelenterazine, whereas the degree of red shift varies per coelenterazine in the non-natural amino acids incapable of H-bonding. The differences in red shift among the coelenterazines in Bromo82AEQ and Iodo82AEQ are more dif-

acs chemical biology

ficult to explain, but it can be observed that, with the exception of coelenterazine *i*, the coelenterazines exhibiting larger red shifts when paired with Bromo82AEQ (*ntv*, *f*, *fcp*, and *cp*) all contain a group (phenol or F) in the C2 position which will allow H-bonding with the aequorin molecule, and the coelenterazines with smaller red shifts (*h*, *ip*, *n*, and *hcp)* do not have this H-bonding capacity. It is possible that steric, aromatic, and heavy atom interactions may also have an affect, yet it is quite likely that these H-bonding factors, in both the non-natural amino acid and the coelenterazine analogues, contribute largely to the observed trend differences in the non-natural aequorins.

While the exact mechanism of bioluminescence in still unconfirmed, changes in the wavelength of bioluminescence have been previously observed due to alteration in the extent of $\pi$ electron delocalization in the chromophore as well as changes in the rigidity of the chromophore. The different H-bonding abilities of the natural and non-natural mutations could be altering the chromophore's electron delocalization or rigidity and, therefore, altering the wavelength of bioluminescence emission (*11, 12*). Another interesting trend when incorporating these non-natural amino acids into aequorin is the unique emission spectral shape of iodo-82-Aeq and bromo-82-Aeq when paired with the coelenterazine analogues containing altered C8 functionality (*cp*, *fcp*, *hcp*, and *ip*). These non-natural aequorins exhibit either a large shoulder in the 420 nm range or a broad and flat spectral shape whose maximum likely combines both the 470 and 420 nm spectral components equally (Figure 2). This may be due to an unusually large population of the neutral coelenteramide species (420 nm) in addition to the anionic I coelenteramide species (470 nm) (*13, 14*).

Additionally, the bioluminescent half-lives of the non-natural aequorin mutants shifted depending on the non-natural amino acid incorporated and the coelenterazine analogue utilized, with bioluminescent half-lives ranging from 0.12 to 14.3 s (see Supplementary Table 7). The most significant half-life shifts occurred with the variation of the coelenterazine analogues (14 s), while, with the exception of coelenterazine *i*, the identity of the non-natural amino acid made far less difference in the bioluminescence half-life of these moieties (see Supplementary Table 7). The half-life shifts observed in these non-natural aequorins also correspond well to the half-life trends previously observed when incorporating these coelenterazine analogues into natural aequorin mutants (*4, 5*). Coelenterazine *i*, in particular, may exhibit its exceptionally long half-life due to a heavy atom effect and may prove to be quite useful in imaging applications due to its extended bioluminescence emission. Lastly, we determined the specific activity of these non-natural aequor-

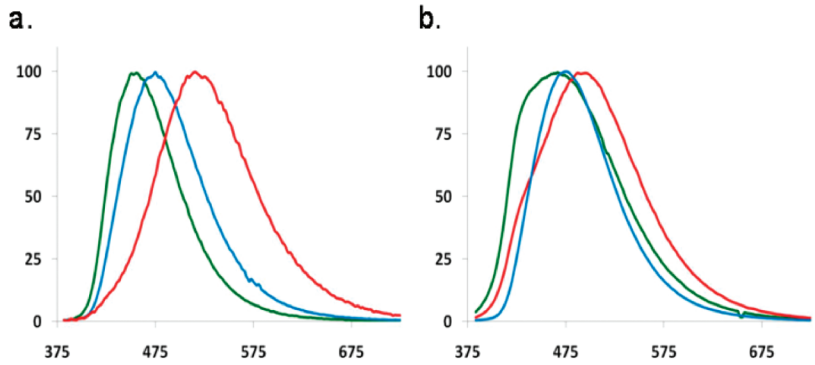



**Figure 2. A) Spectral differences of natural versus non-natural aequorin bioluminescence (green = cysteine free aequorin + coelenterazine *cp*, blue = cysteine-free aequorin + coelenterazine *native*, red = methoxy-82-aequorin + coelenterazine *i*. B) Altered spectral shape of bromo-82-aequorin (green = bromo-82-aequorin + coelenterazine *cp*, red = bromo-82-aequorin + coelenterazine *native*, blue = cysteine-free aequorin + coelenterazine *native*).**

**TABLE 1. Emission maxima (nm) of aequorin with non-natural amino acids with coelenterazine analogues[a]**

| Coelenterazine | Aequorin-cys free | Amino82AEQ | Bromo82AEQ | Iodo82AEQ | Methoxy82AEQ |
|---|---|---|---|---|---|
| Native | 472 | 483 | 491 | 487 | 509 |
| *i* | 484 | 496 | 504 | 493 | 517 |
| *cp* | 454 | 466 | 465 | 470 | 489 |
| *f* | 480 | 492 | 493 | 490 | 515 |
| *fcp* | 463 | 477 | 475 | 476 | 498 |
| *h* | 472 | 484 | 480 | 482 | 509 |
| *hcp* | 454 | 466 | 458 | 465 | 488 |
| *ip* | 454 | 465 | 461 | 465 | 485 |
| *n* | 475 | 485 | 481 | 485 | 511 |

[a]Aeq Cys-free is the baseline mutant used, in which all three native Cys residues have been mutated to Ser. Amino82AEQ is aequorin with 4-amino-Phe substituted for Tyr at position 82, Bromo82AEQ is aequorin with 4-bromo-Phe substituted for Tyr at position 82, etc.

ins (see Supporting Information) and found that, although the bioluminescent intensities of our non-natural aequorins are lower than the native form, they are strong enough to be of utility in assay- and cell-based applications.

In the future we plan on incorporating additional non-natural amino acids into aequorin in order to further shift the emission maxima as well as undertaking a more thorough characterization of these proteins. We also plan on incorporating non-natural amino acids containing groups such as benzophenones and ketones into aequorin in order to increase the utility of aequorin for *in vivo* applications such as protein−protein interaction studies. The long-term goal of this project is to use these non-natural bioluminescent proteins to track multiple cellular processes simultaneously by taking advantage of the spectral and temporal resolution and the photocross-linking ability and unique chemical "handles" that site-specific incorporation of non-natural amino acids allows. The success in achieving significant emission shifts and spectral shape alterations in aequorin highlights the promise of non-natural amino acid incorporation in tuning the bioluminescence properties and diversity of not only other bioluminescent proteins, such as firefly and Renilla luciferase, but the properties of other reporter proteins as well (*15, 16*). We envision that the ability to rationally tailor and incorporate non-natural amino acids with unique functionalities into bioluminescent proteins in order to fine-tune their emission characteristics will create unique designer proteins with distinct characteristics. The plethora of unique functionalities that non-natural amino acids offer will greatly increase the breadth of applications to which the newly created bioluminescent reporter proteins are suited, including multimodal imaging and multiplexing technologies, spatial and temporal control of bioluminescent reporters *in vivo*, and bioluminescence resonance energy transfer (BRET) studies (*17–19*).

## METHODS

**Reagents.** The qwik-change site-directed mutagenesis kit was purchased from Stratagene, the oligonucleotide primers from Eurofins MWG Operon, the pBADHisA plasmid from Invitrogen, and the restriction enzymes from either NEBiolabs or Promega. The T4 DNA ligase and T4 DNA ligase buffer were purchased from Promega, the exTaq DNA polymerase from Takara, and the plasmid DNA purification and gel extraction kits from Qiagen and tris(hydroxymethyl)amino methane (Tris) free base, ethylenediaminetetraacetic acid (EDTA) sodium salt, glucose, sodium dodecyl sulfate (SDS), ampicillin, tetracycline, and arabinose were purchased from Sigma. All other components necessary for arabinose autoinduction media were obtained from Sigma or DIFCO. Glacial acetic acid was purchased from EM Science and all coelenterazines from Biotium, and all non-natural amino acids were from PepTech. SDS-PAGE precast gels were purchased from Invitrogen. Composition of different buffers used were as follows: buffer A: 30 mM Tris−HCl containing 2 mM EDTA, pH 7.0; buffer B: 30 mM Tris−HCl containing 2 mM EDTA, 1 mg $mL^{-1}$ BSA, and 150 mM NaCl, pH 7.0; buffer C: 30 mM Tris−HCl and 100 mM $CaCl_2$, pH 7.0. All chemicals were reagent grade or better, and the solutions were prepared using deionized (Milli-Q water purification system, Millipore) reverse-osmosis purified water.

**Apparatus.** Protein was expressed by incubating bacteria in a Fisher Scientific orbital shaker incubator. Cell cultures were centrifuged in a Beckman J2-MI centrifuge. Purity of the protein was examined with Coomassie Blue staining with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Novex mini-cell apparatus from Invitrogen. Aequorin activity was measured using an Optocomp I luminometer from MGM Biomedical. The emission spectra of the aequorin−coelenterazine pairs were taken on a custom-made SpectroScan instrument from ScienceWares, which is capable of obtaining spectra from flash reactions of luminescent samples that emit in the 400−700 nm range.

**Mutation of Aequorin and Cloning into pBADHisA.** The 82TAG aequorin mutant was prepared using the qwik-change site-directed mutagenesis kit and the following primers: 5′-GGAAACTGATTGGCCTGCATAGATTGAAGGATGG-3′ and 5′-CCATCCTTCAATCTATGCAGGCCAATCAGTTTCC-3′. This mutagenesis changed the codon for tyrosine (TAT) at position 82 in aequorin to a TAG stop codon. The template for the mutatgenesis reaction was pIN4 containing the cysteine-free aequorin. The mutant aequorin gene along with the ompA leader sequence from the pIN4 was amplified by PCR placing an Nco I restriction site at the 5′ end and a Hind III site at the 3′ end. The sequence of the forward primer for this PCR was 5′-CCATGGGTATGAAAAAGACAGCTATCGCGATTGC-3′, while the reverse primer had the sequence 5′-AAGCTTAGGGGACAGCTCCACCGTAGAGCTTTTCGGAAGCAGGATCCATTGTGTAC-3′. The PCR was carried out using ExTaq polymerase with the following PCR parameters: 94 °C for 2 min, followed by 30 cycles of 94 °C × 30 s, 55 °C × 30 s, 72 °C × 60 s, and ending with 72 °C for 7 min. The PCR product was inserted into the Nco I and Hind III sites of pBAD/HisA to produce pBADHisA−A82EQ82TAG. Transformation of *E. coli* with this plasmid produced a system in which the expression of aequorin is regulated by the addition of arabinose to the culture medium. Expression of aequorin with the ompA leader sequence results in the protein being released into the culture medium.

**Construction of the pDULE-pBADHisA−A82EQ82TAG Expression Strains.** Four pDULE vectors which allow for the site-specific incorporation of four different non-natural amino acids were obtained from Dr. Peter Schultz (Scripps Research Institute) and Dr. Ryan Mehl (Franklin and Marshall College) (*2*). Each of these pDULE plasmids coded for a tRNA and tRNA synthetase

specific for a TAG codon and a single non-natural amino acid, either L-4-aminophenylalanine, L-4-bromophenylalanine, L-4-iodophenylalanine, or L-4-methoxyphenylalanine. These plasmids were transformed into chemically competent MAXEfficiency DH10B *E. coli* cells and transformants selected by plating on LB agar medium containing tetracycline (12 μg $mL^{-1}$). Following confirmation of the transformation *via* plasmid isolation, restriction enzyme digestion and DNA gel electrophoresis, the DH10B *E. coli* cells containing the pDULE plasmids were made chemically competent using the standard rubidium chloride method and transformed with pBADHisA−AEQ82TAG to produce four different strains, each specific for the incorporation one of the non-natural amino acids mentioned above. Selection for these transformants was by plating on LB agar medium containing both tetracycline (12 μg $mL^{-1}$) and ampicillin (100 μg $mL^{-1}$).

***in Vivo* Expression and Purification of Aequorin with Non-Natural Amino Acids Site-Specifically Incorporated at Position 82.** Cells were grown in 500 mL of autoinduction medium consisting of M9 minimal salts medium containing 0.5% glycerol, 0.048% glucose, 0.05% arabinose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, leucine (80 μg $mL^{-1}$), 5 mL of 100 X trace metals (see below), ampicillin (50 μg $mL^{-1}$), and tetracycline (6 μg $mL^{-1}$).

100 X Trace Metals

boric acid, 0.57 g
copper(II) sulfate pentahydrate, 0.39 g
ferric chloride (100 g in 40 mL water), 2.0 mL
manganese chloride tetrahydrate, 4.0 g
sodium chloride, 5.0 g
sodium molybdate dihydrate, 0.5 g
magnesium sulfate heptahydrate, 25.0 g
sulfuric acid, 2.87 mL
zinc sulfate heptahydrate, 1.0 g

The above were dissolved in 1 L of $H_2O$ and filtered through a 0.22 μm filter. The inoculum was prepared by growing cells overnight in 25 mL LB + ampicillin (50 μg $mL^{-1}$) and tetracycline (6 μg $mL^{-1}$) at 37 °C × 250 rpm. The cells were pelleted and resuspended in 10 mL of the autoinduction medium and then added to 500 mL of autoinduction medium. Inoculated flasks were incubated at 37 °C × 250 rpm. When the optical density of the culture reached 0.2−0.4, the non-natural amino acid was added to 1 mM final concentration. Depending on the solubility of the non-natural amino acid, the amino acid was first added to ~50 mL of autoinduction medium which was gently heated until the amino acid was dissolved. The dissolved non-natural amino acid was then filtered through a 0.22 μm filter before adding to the culture. The culture was then allowed to grow at 37 °C × 250 rpm overnight.

**Purification of Aequorin.** The cells were pelleted at 12 000 g × 20 min, and the supernatant was then transferred to a clean flask. The pH of the supernatant was adjusted to 4.3 by the slow dropwise addition of glacial acetic acid with stirring. The precipitated protein was then pelleted by centrifugation at 20 000 g × 20 min. The pellet was resuspended in 35 mL of 30 mM Tris−HCl, pH 7.5, and 2 mM EDTA, and the pH was adjusted to 7.5 by the dropwise addition of 1 N NaOH. The protein was recentrifuged at 20 000 × g for 20 min to remove particulates before purification. See ref 4 for purification protocol. Purified apoaequorin was then sterilely filtered through a 0.2 μM filter before storage at either 4 °C for short-term storage or −20 °C for long-term storage.

**Mass Spectrometry.** Proteins were run on SDS-PAGE and cut out of the stained gels. Gel pieces were digested with trypsin, and LC-ESI-MS-MS performed using a ThermoFinnigan LTQ. Resulting MS-MS spectra were searched against proteins in the SwissProt database using the X!Tandem search engine.

**Determination of Aequorin Activity.** Aequorin activity was determined using an Optocomp I luminometer. Aequorin was diluted with 30 mM Tris−HCl, pH 7.5, 2 mM EDTA, and 0.5% bovine serum albumin to ensure the amount of light produced was within the linear range of the instrument. Activity was triggered by injecting 50 μL of 100 mM Tris−HCl, pH 7.5, and 100 mM $CaCl_2$. Bioluminescence was counted at 0.1 s intervals for a total of 6 s.

**Emission Spectra of Photoproteins.** All emission spectra were produced using the SpectroScan luminometer from ScienceWares. The non-natural aequorins were first charged by incubating overnight at 4 °C with a five-fold molar excess of each of the different coelenterazines. Each charged non-natural aequorin was pipetted into a 96-well microtiter plate and scanned from 400−700 nm using the custom built CCD luminometer, following injection of 100 μL of buffer C. For each well, the CCD took readings for 10 s, scanning an array of wavelengths from 400−700 nm with an interval of 1.5 nm. All photoprotein spectra were measured in triplicate and blank corrected, and the mean of these three values were reported. A five to seven point moving average was also applied to select spectra in order to smooth the curves; this averaging did not alter the final emission maxima.

**Half-life Determination of Photoproteins.** Non-natural aequorins were charged overnight with the appropriate coelenterazine analogue and then diluted with 100 mM Tris−HCl, pH 7.5, buffer until the maximum counts on the luminometer were between 5000 and 50 000 RLU's in order to not overload the PMT detector. A Polarstar 96-well microplate reading luminometer (BMG Labtech) was utilized with the bioluminescence signal of a 50 μL sample and was measured for 30 s following the injection of 100 μL of triggering buffer (100 mM Tris−HCl, pH 7.5, and 100 mM $CaCl_2$). All

measurements were done in triplicate and blank subtracted. The mean bioluminescence decay spectra was fit with an exponential decay equation using GraphPad Prism 4.0 (GraphPad Software), and a first-order decay kinetics half-life equation was used to determine the bioluminescence half-life of each aequorin−coelenterazine pair.

*Acknowledgment:* S.Daunert would like to thank the University of Kentucky for a University Research Professorship and a Gill Eminent Professorship. L.Rowe acknowledges funding from the National Science Foundation-Integrative Graduate Education and Research Traineeship Program and the National Institutes of Health-Predoctoral Fellowships. This work was funded by a grant from the National Institutes of Health (GM 467917). The authors are grateful to P. Schultz from the Scripps Research Institute for his generous donation of pDULE and pBK plasmids.

*Supporting Information Available:* This material is available free of charge *via* the Internet at http://pubs.acs.org.

## REFERENCES

1. Hutchens, M., and Luker, G. (2007) Applications of bioluminescence imaging to the study of infectious diseases, *Cell. Microbiol. 9*, (10), 2315–2322.
2. Dikici, E., Qu, X., Rowe, L., Millner, L., Logue, C., Deo, S. K., Ensor, C. M., and Daunert, S. (2009) Aequorin variants with improved bioluminescent properties, *Protein Eng., Des. Sel. 22*, (4), 243–248.
3. Prendergast, F. G. (2000) Bioluminescence illuminated, *Nature 405*, (6784), 291–293.
4. Rowe, L., Dikici, E., Logue, C., Deo, S. K., Ensor, C. M., and Daunert, S. (2009) Spectral tuning of photoproteins by partnering site-directed mutagenesis strategies with the incorporation of chromophore analogues, *Protein Eng., Des. Sel. 21*, (1), 73–81.
5. Shimomura, O., Musicki, B., and Kishi, Y. (1988) Semi-synthetic aequorin: An improved tool for the measurement of calcium ion concentration, *Biochem. J. 251*, (2), 405–410.
6. Vysotski, E. S., and Lee, J. (2004) Ca2+ regulated photoproteins: structural insight into the bioluminescence mechanism, *Acc. Chem. Res. 37*, 405–415.
7. Stepanyuk, G. A., Golz, S., Markova, S. V., Frank, L. A., Lee, J., and Vysotski, E. S. (2005) Interchange of aequorin and obelin bioluminescence color is determined by substitution of one active site residue of each photoprotein, *FEBS Lett. 579*, 1008–1014.
8. Wang, L., Brock, A., Herberich, B., and Schultz, P.G. (2001) Expanding the genetic code of *E. coli*, *Science 20*, (5516), 498–500.
9. Farrell, I. S., Toroney, R., Hazen, J. L., Chin, J. W., and Mehl, R. A. (2005) Photo-cross-linking interacting proteins with a genetically encoded benzophenone, *Nat. Methods 2*, (5), 377–384.
10. Jackon, J. C., Duffy, S. P., Hess, K. R., and Mehl, R. A. (2006) Improving nature's enzyme active site with genetically encoded unnatural amino acids, *J. Am. Chem. Soc. 128*, (34), 11124–7.
11. Branchini, B., Southworth, T. L., Murtiashaw, M. H., Magyar, R. A., Gonzalez, S. A., Ruggiero, M. C., and Stroh, J. G. (2004) An alternative mechanism of bioluminescence color determination in firefly luciferase, *Biochemistry 43*, 7255–7262.
12. Nakatsu, T., Ichiyama, S., Hiratake, J., Saldhna, A., Kobashi, N., Sakata, K., and Kato, H. (2006) Structural basis for the spectral difference in luciferase bioluminescence, *Nature 440*, 372–376.
13. Belogurova, N. V., Kudryasheva, N. S., Alieva, R. R., and Sizykh, A. G. (2008) Spectral components of bioluminescence of aequorin and obelin, *J. Photochem. Photobiol., B 92*, (2), 117–122.
14. Vysotski, E. S., *et al.* (2003) Violet bioluminescence and fast kinetics from W92 obelin: structure based proposals for the bioluminescence triggering and the identification of emitting species, *Biochemistry 42*, 6013–6024.
15. Viviani, V. R. (2002) The origin, diversity, and structure function relationships of insect luciferases, *Cell. Mol. Life Sci. 59*, 1833–1850.
16. Ohmiya, Y. (2005) Basic and applied aspects of color tuning bioluminescence systems, *Jpn. J. Appl. Phys. 44*, (9A), 6368–6379.
17. Xie, J., and Schultz, P. G. (2005) Adding amino acids to the genetic repertoire, *Curr. Opin. Chem. Biol. 9*, (6), 548–554.
18. Kim, S. B., Otani, Y., Umezawa, H., and Tao, H. (2007) Bioluminescent indicator for determining protein−protein interactions using intramolecular complementation of split click beetle luciferase, *Anal. Chem. (Washington, DC, U. S.) 79*, (13), 4820–4826.
19. Prinz, A., Reither, G., Diskar, M., and Schultz, C. (2008) Fluorescence and bioluminescence procedures for functional proteomics, *Proteomics 8*, 1179–1196.