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Spectral tuning of photoproteins by partnering site-directed mutagenesis strategies with the incorporation of chromophore analogs

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Aequorin and obelin are photoproteins whose calcium controlled bioluminescent light emission is used for labeling in assays, for the determination of calcium concentrations *in vivo*, and as a reporter in cellular imaging. Both of these photoproteins emit blue light from a 2-hydroperoxycoelenterazine chromophore, which is non-covalently bound in the hydrophobic core of the proteins. In an effort to produce aequorin and obelin variants with improved analytical properties, such as alternative emission colors and altered decay kinetics, seven mutants of aequorin and obelin were prepared and combined with 10 different coelenterazine analogs. These semi-synthetic photoprotein mutants exhibited shifts in bioluminescent properties when compared with wild-type proteins. The bioluminescent parameters determined for these semi-synthetic photoprotein mutants included specific activity, emission spectra and decay half-life time. This spectral tuning strategy resulted in semi-synthetic photoprotein mutants that had significantly altered bioluminescent properties. The largest emission maxima shift obtained was 44 nm, and the largest decay half-life difference was 23.91 s.

Keywords: aequorin/bioluminescence/cysteines/obelin/photoproteins

Introduction

Light-emitting proteins are integral to a wide variety of scientific techniques, from the development of miniaturized biosensors to the non-invasive imaging of cellular and sub-cellular events and mechanics (Daunert and Deo, 2006). Both fluorescent and bioluminescent proteins are utilized in these techniques, and a thriving field of research involves the molecular tuning of these light-emitting proteins for the purpose of optimizing their physical properties. The physical properties that such experiments aim to alter include the stability, activity, aggregation tendency and cellular permeability, as well as the light-emitting properties of the proteins, such as excitation and emission wavelengths and decay kinetics. The properties of fluorescent proteins such as GFP and DsRed have been extensively altered using both site-directed and random mutagenesis (Labas *et al*., 2002), so that researchers now have a diverse collection of fluorescent proteins to choose from when designing an experiment (Giepmans *et al*., 2006). However, despite the spectral diversity and stability that these now afford, the use of fluorescent proteins has disadvantages for certain experiments. Thus, a significant push in recent years has been focused on tuning the properties of bioluminescent proteins (Nakatsu *et al.,* 2006), which can be more sensitive in biological fluids than fluorescent proteins. Molecular tuning efforts have been focused on both major bioluminescent superfamilies, the adenylation enzymes common in fireflies and the calcium-binding proteins common in jellyfish (Ohmiya, 2005). In this article, we describe the tuning of two calcium-binding bioluminescent photoproteins, aequorin and obelin, by first mutating the proteins and then pairing these mutants with altered, exogenous chromophores.

Aequorin is a 22 kDa, 189 amino acid protein which was originally isolated from the marine jellyfish *Aequorea victoria* (Shimomura, 1995), whereas obelin is a 22 kDa, 192 amino acid protein isolated from the hydroid *Obelia longissima* (Illarionov *et al*., 1995). Aequorin and obelin have both been successfully cloned into bacterial hosts (Inouye *et al.,* 1985; Illarionov *et al*., 2000) and have had their X-ray crystal structures solved (Head *et al*., 2000, Liu *et al*., 2000, 2003, 2006; Deng *et al*., 2004). These structures reveal that aequorin and obelin exhibit high structural homology to one other, both being globular proteins that have a central, hydrophobic pocket in which a coelenterazine chromophore tightly, but non-covalently, binds. Coelenterazine, an imidazopyrazinone molecule, is sequestered in the hydrophobic cavity of aequorin and obelin in a 2-hydroperoxycoelenterazine state (Prendergast, 2000; Vysotski and Lee, 2005). Calcium binding to the three Ca^{2+}-binding EF hand loops of aequorin and obelin causes a slight conformational change which leads to the oxidation of the hydroperoxycoelenterazine to an excited coelenteramide. The relaxation of this coelenteramide proceeds radiatively, producing the bioluminescence of both aequorin and obelin (471 and 491 nm, respectively).

Thus far, the spectral tuning of both aequorin and obelin has been accomplished primarily by site-directed and random mutagenesis. By employing these methods, significant improvements have been made in the wild-type proteins, including enhanced activity and thermostability, altered decay kinetics and the development of several new emission colors (Ohmiya *et al*., 1992; Malikova *et al*., 2003; Stepanyuk *et al*., 2005; Tricoire *et al*., 2006; Tsuzuki *et al*., 2005). Additionally, previous studies with wild-type aequorin showed that altering the structure of coelenterazine in specific locations allows for the retention of activity, but simultaneously causes spectral shifts and changes in the bioluminescent decay kinetics (Shimomura *et al*., 1988, 1989, 1990, 1993; Inouye and Shimomura, 1997; Hirano *et al*., 1998; Zheng *et al*., 2000). Therefore, we envisioned that pairing the technique of site-directed mutagenesis with the incorporation of altered chromophores would significantly increase the likelihood of developing photoproteins with novel and improved bioluminescent properties.

Materials and methods

Reagents

Tris(hydroxymethyl)amino methane (Tris) free base, ethylenediaminetetraacetic acid (EDTA) sodium salt, glucose, sodium dodecyl sulfate (SDS), ampicillin and all other reagents were purchased from Sigma (St Louis, MO, USA). Luria Bertrani (LB) Broth was obtained from DIFCO (Sparks, MD, USA). Glacial acetic acid was purchased from EM Science (Gibbstown, NJ, USA). POROS Selfpack 20 HQ strong anionic exchange beads for column packing were from Applied Biosystems (Foster City, CA, USA). All coelenterazines were purchased from Biotium (Hayward, CA, USA). 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 and 2 mM DTT, pH: 7.0; buffer C: 30 mM Tris–HCl containing 2 mM EDTA and 1 M NaCl, pH: 7.0; buffer D: 30 mM Tris–HCl containing 2 mM EDTA, 2 mM DTT and 1 M NaCl, pH: 7.0 and buffer E: 100 mM $CaCl_2$ with 30 mM Tris–HCl. All chemicals were reagent grade or better and solutions were prepared using deionized (Milli-Q Water Purification System, Millipore, Bedford, MA, USA) distilled water.

Apparatus

Protein was expressed by incubating bacteria in an orbital shaker at 37°C in a Fisher Scientific incubator (Fair Lawn, NJ, USA) and centrifuged in a Beckman J2-MI centrifuge (Palo Alto, CA, USA). Aequorin and obelin mutants were purified using a BioCAD SPRINT Perfusion Chromotography System from PE Biosciences (Framingham, MA, USA). Purity of the protein was checked with Coomassie Blue staining with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Activity measurements during purification, and for specific activities, were taken on an Optocomp 1 luminometer from MGM Biomedical (Carrborro, NC, USA). Half-life measurements were taken on a Polarstar Optima luminometer from BMG Labtech (Durham, NC, USA). All luminescence intensities reported are the average of a minimum of three replicates and have been corrected for the contribution of the blank. The emission spectra of the semi-synthetic photoprotein mutants were taken on a custom made SpectroScan instrument from ScienceWares (Framingham, MA, USA), which is capable of obtaining spectra from flash reactions of luminescent samples that emit in the 400–700 nm range. This instrument was based on a Thermo-Labsystems Luminoskan Ascent luminometer and accommodates 96-well microliter plates. The light from the flash reaction is detected by the circular end of a circle-to-line quartz fiber optic bundle. The line end of this bundle is used as the entrance slit to a high throughput imaging spectrograph, which is monitored by a cooled, back-thinned, andor CCD camera. The dispensing of $CaCl_2$ occurs after CCD integration has begun.

Methods

Expression and purification of aequorins

All mutant aequorins and obelins were prepared using Qwikchange site-directed mutagenesis kits. The aequorin mutants included a cysteine-free aequorin (Cys-free Aeq) in which all three native cysteine residues were mutated to serines, and three single cysteine aequorins. The Cys-free aequorin had amino acids at positions 5, 53 and 71 (Aeq S5C, Aeq E53C and Aeq M71C, respectively) mutated to cysteine in order to produce the three single cysteine aequorin mutants (Lewis *et al*., 2000). In addition, wild-type obelin (WT-Obl) had four of its five native cysteine residues altered to serine residues. The two single obelin mutants had a cysteine at positions 151 and 158 altered to serines (Obl C151S and Obl C158S). An obelin double mutant was also produced by mutating both cysteines, at positions 67 and 75, to serines, to produce Obl C67+75S. Following confirmation of the mutation with DNA sequencing, the DNA sequences coding for the mutant photoproteins and the wild-type photoproteins (WT-Aeq and WT-Obl) were digested and ligated into a pin4 plasmid containing an OmpA leader sequence, an lpp promoter and an ampicillin resistance gene. Glycerol stock of all mutants and wild-type photoproteins was prepared with overnight culture grown in LB Broth containing 100 μg/ml ampicillin. The plasmids were then transformed into Top10 *Escherichia coli* cells and expressed in the following manner. Two to three sterilized toothpicks of −80°C stored glycerol stock was added to 500 ml of LB Broth (20 g powder/l solution) containing 100 μg/ml ampicillin in 1 l flasks. The cultures were orbitally shaken at 37°C for ∼16 h until an OD_{600} of 0.4–0.6 was reached, then 1 mM IPTG was added to the flask in order to induce expression. The protein was expressed for 16 h at 37°C with 250 rpm orbital shaking. With this expression system, the photoprotein was released into the medium. Cultures were then centrifuged at 4°C, 12 000*g* for 20 min to pellet the cells. The supernatant was kept and the pellet discarded. The supernatant was acid precipitated by decreasing the pH to 4.2 with glacial acetic acid and stirring at 4°C overnight, followed by centrifugation at 12 000*g*, 4°C for 30 min, discarding the supernatant and keeping the pellet. The pellet was resuspended in 35 ml of 30 mM Tris/HCl, pH 7.5, 2 mM EDTA buffer and the pH was adjusted to 7.5 using 1 N NaOH. The protein solution was then filtered through a 0.2 μm filter. The apoproteins were then purified by chromatography on Poros HQ (Applied Biosystems) and Butyl Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden) columns using a BioCad Sprint system (Applied Biosystems). The protein solution was applied to a Poros HQ column (20 mm × 65 mm) equilibrated with a 30 mM Tris/HCl, pH 7.5, 2 mM EDTA buffer and eluted with a 0–50% gradient of 30 mM Tris/HCl, pH 7.5, 2 mM EDTA, 1 M NaCl buffer over 10 column volumes. The most active fractions were pooled together and ammonium sulfate was added to a 1 M final concentration. This protein solution was then applied to a Butyl Sepharose Fast Flow column (16 mm × 90 mm) equilibrated with 20 mM Bis–Tris, pH 7.5, 2 mM EDTA, 1 M ammonium sulfate and eluted with a 0–80% gradient of 20 mM Bis–Tris, pH 7.5, 2 mM EDTA buffer. Fractions were analyzed for activity and purity (>95%) using SDS–polyacrylamide gels, western blots and by assaying for activity. Fractions showing high UV absorbance and luminescent activity were combined and dialyzed against buffer B overnight. Protein samples were checked for purity using SDS–PAGE, Coomassie Blue staining, and all of them showed a single band corresponding to the molecular weight of apoaequorin or apoobelin. Protein concentrations were determined using

optical density, with triplicate measurements averaged and blank corrected, for final concentration. All proteins were stored in the dark at 4°C in the refrigerator in air tight containers following purification.

Determining the specific activity of the photoproteins

All coelenterazines were diluted to 100 μg coelenterazine/ml with methanol after the methanol had been deoxygenated by passing nitrogen gas through it for 15 min. Coelenterazines were desiccated and stored in a light free, −80°C, freezer until needed. The activity of aequorin was determined by vortexing a volume of 100 μl of apoprotein solution with 3 μl native coelenterazine in an Eppendorf tube. This solution was incubated overnight at 4°C in order to completely charge the apoprotein. Ten microliter of this solution was assayed for activity in the Optocomp 1 luminometer by injecting 100 μl of buffer E and integrating the light signal over a 6 s period. All reported data are the mean of three replicates, corrected for the blank.

Emission spectra of photoproteins

All emission spectra were produced using the SpectroScan luminometer from ScienceWares. The apoprotein was first charged with the appropriate coelenterazine by incubating it overnight at 4°C with a 5-fold molar excess of the coelenterazine analog. The charged photoprotein was then diluted with buffer B until it generated a luminescent activity range between 1 and 3 million relative light units (RLU) in order to not overload the CCD detector while minimizing spectral noise. A volume of 50 μl of the charged photoprotein was pipetted into a 96-well microliter plate and scanned from 400 to 700 nm using the custom built CCD luminometer, following injection of 50 μl of buffer E. For each well, the CCD took readings for 5 s, simultaneously recording an array of wavelengths from 400 to 700 nm, with an interval of 1.5 nm. All photoprotein spectra were measured in triplicate, blank corrected, and the mean of these three values were reported. A five-point moving average was also applied to select spectra in order to smooth the curves, this averaging did not alter the emission maxima.

Determination of half-life

All decay half-life times were determined using the Polarstar luminometer. The apoprotein was charged with the appropriate coelenterazine in the same manner as described in Materials and methods, detailing how the specific activity of the photoproteins was determined. The charged photoprotein was then diluted with buffer B until a maximum activity reading between 5000 and 50 000 RLUs was reached, and 50 μl of this was pipetted into a microliter plate well. A volume of 100 μl of buffer E was then added to photoprotein solution and light was collected over a 10 s time period, at intervals of 0.04 s. Half-life determinations reported were the average of triplicates, and blank subtracted. Data were analyzed with GraphPad Prism 4.0. Initial increasing bioluminescent data points were excluded from the half-life calculation in order to analyze only the decay of aequorin. The bioluminescent decay curve was fit with a non-linear regression, one-phase exponential decay equation, with the plateau constrained to equal zero. The half-life was determined using a first order decay kinetics equation.

Results

In this study, wild-type aequorin and obelin (WT-Aeq and WT-Obl) have had their native cysteine residues altered, and/or additional cysteine residues added. WT-Aeq contains three cysteine residues, at positions 145, 152 and 180. The mutants herein were constructed in our laboratory, and the aequorin mutants consisted of a cysteine-free aequorin (Cys-free Aeq), in which all three native cysteine residues are mutated to serines, and three mutants which contained a single cysteine residue. The single cysteine aequorin mutants were constructed by mutating a single amino acid at positions 5, 53 and 71 on the Cys-free Aeq template to a cysteine residue (Cys-free Aeq, Aeq S5C, Aeq E53C and Aeq M71C) (Lewis *et al.*, 2000) (Fig. 1). WT-Obl contains five cysteine residues,

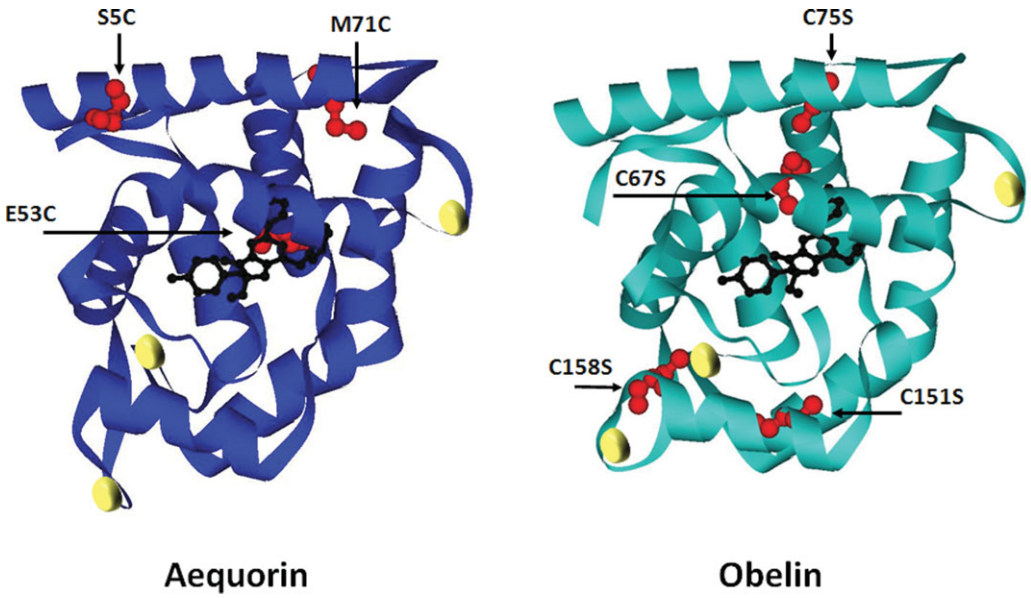


Fig. 1. The X-ray crystal structure of WT-Aeq and WT-Obl. The coelenterazine chromophore is shown in black, calcium ions are shown as yellow ovals and mutation sites are shown in red.

at positions 51, 67, 75, 151 and 158. The obelin mutants consisted of two mutants that had one of the native cysteine residues mutated to a serine, at positions 151 and 158 (Obl C151S, Obl C158S), and a double mutant that had two cysteine residues mutated to serine residues, at position 67 and 75 (Obl C67+75S) (Fig. 1).

The specific activities of the semi-synthetic photoprotein mutants, and WT-Aeq and WT-Obl, when charged with coelenterazine *ntv*, are summarized in Table I. Results indicate that there is a 3× and 1.5× increase of bioluminescence activity, when compared with WT-Aeq, in the Cys-free Aeq and Aeq E53C, respectively. Activity was reduced by approximately an order of magnitude in Aeq S5C and Aeq M71C. All of the obelin mutants lost a significant amount of activity when compared with wild-type. Obl C158S lost the most activity of any photoprotein mutant, decreasing in activity by over two orders of magnitude.

The emission spectra and decay half-life time of all nine of these photoproteins was then determined when each mutant was paired with 10 coelenterazine analogs (Table II). The emission maxima for the seven photoprotein mutants and two wild-type photoproteins, containing the 10 different coelenterazines, are given in Table III. The 20 nm difference in the emission maxima of WT-Aeq and WT-Obl when paired with native coelenterazine (ctz *ntv*) concurs with previous studies (Stepanyuk *et al*., 2005). The largest difference in emission maxima among all of the semi-synthetic photoprotein mutants is 44 nm, between the Cys-free Aeq, Aeq E53C and Aeq M71C paired with coelenterazine *ip*, *cp* and *hcp* ($\lambda = 453$ nm) and Aeq M71C, WT-Obl and the double mutant Obl C67+75S paired with coelenterazine *i* ($\lambda = 498$, 497, 497 nm). Figure 2 shows the significant separation of the emission maxima between two of these semi-synthetic photoprotein mutants. The largest emission maxima difference between all of the photoproteins and a single coelenterazine analog is 20 nm (Fig. 2), between the WT-Aeq ($\lambda =$ 471 nm) and WT-Obl and the double mutant Obl C67+75S ($\lambda = 491$ nm). Further analysis of the data shows that the incorporation of coelenterazine *ip*, *cp* and *hcp* into the photoprotein mutants all result in significant blue shifts, whereas coelenterazine *i* and *f* both cause large red shifts. The other coelenterazine analogs, *h*, *fcp*, *e* and *n* cause emission maxima shifts to a much lesser degree. The individual mutations on the photoproteins also altered the effect of the coelenterazine analog. For example, WT-Obl and the double mutant Obl C67+75S yielded emission maxima of 491 nm when paired with coelenterazine *i*, whereas Obl C151S and Obl C158S both gave blue shifted emission maxima of 476 nm when paired with coelenterazine *I* (Fig. 3). The obelin mutants behaved in the same manner when paired with coelenterazine *h*, *e* and *n*, with WT-Obl and Obl C67+75S emitting at a redder wavelength than either Obl C151S or Obl C158S.

The decay half-lives of the seven mutated photoproteins, and the WT-Aeq and WT-Obl, are listed in Table IV. The largest half-life difference among the mutant photoproteins is 23.91 s, between Aeq S5C paired with coelenterazine *i* (24.07 s) and Obl C151S paired with coelenterazine *h* (0.16 s). Figure 4 shows the significant difference between the decay profiles of two such semi-synthetic photoprotein mutants. There is a noticeably increased half-life in all of the mutants, as well as in the wild-type proteins, when coelenterazine *i* is incorporated (4.20–24.07 s). Coelenterazine *f*, gave only a slightly extended half-life, and only with the aequorin mutants (0.44–0.85 s). The incorporation of coelenterazine *n* resulted in a significant extension in the decay half-lives of all the photoproteins (1.06–10.91 s). The half-life trends of all of the photoprotein mutants when paired with the coelenteraine analogues were very similar to each other, with one exception. The double mutant, Obl C67+75S, yielded much longer half-lives when paired with coelenterazine *ntv*, coelenterazine *ip*, *e* and *n* than any of the other photoproteins (1.83, 8.85, 1.88 and 10.91 s, respectively).

Table I. Specific activities of aequorin and obelin mutants when paired with coelenterazine *ntv*

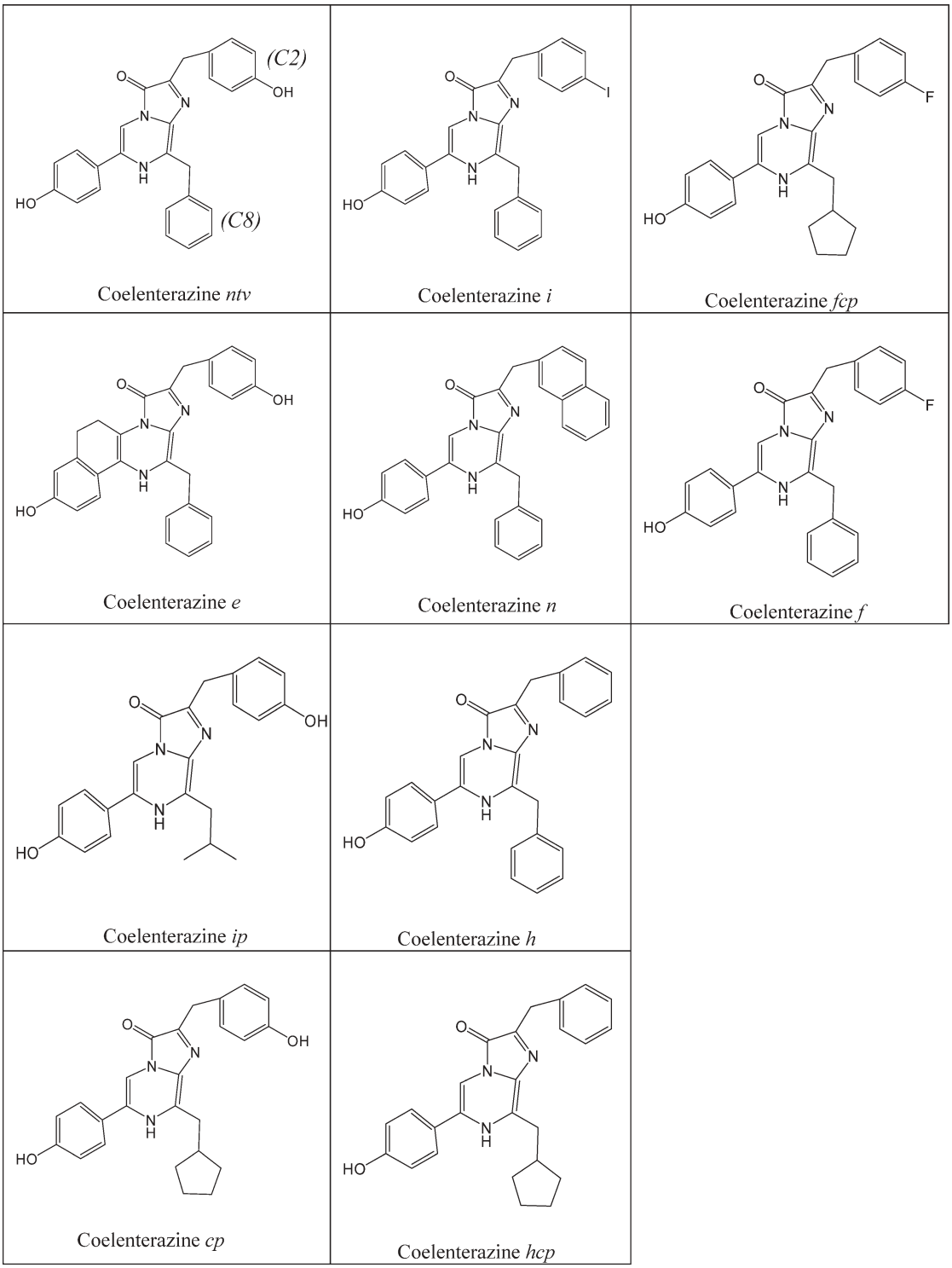
Photoprotein mutant	Specific activity (RLU/mg)	Distance from mutation site(s) to Chromophore(Å)
WT-Aeq	6.7×10^{11}	NA
Cys-free Aeq	2×10^{12}	13.62, 16.00, 13.62
Aeq S5C	1×10^{11}	19.23
Aeq E53C	1×10^{12}	18.71
Aeq M71C	4×10^{11}	17.19
WT-Obl	5.7×10^{12}	NA
Obl C151S	1.4×10^{10}	13.82
Obl C158S	1.1×10^{9}	16.05
Obl C67+75S	6.8×10^{10}	10.60, 14.61

All results are the mean of triplicate measurements, corrected for the blank. Also listed is the distance, in Angstroms, from the carboxylic acid carbon of the mutated amino acid(s) to the C3 carbon of coelenterazine, as measured with wild-type aequorin or wild-type obelin.

Discussion

In an effort to develop photoprotein variants with improved analytical properties we first mutated both aequorin and obelin and then combined these mutants with a variety of coelenterazine analogues. Our mutations focused on the relevance of cysteine residues in both aequorin and obelin, and the development of single cysteine photoprotein variants. From an analytical perspective, single cysteine protein labels offer the significant advantage of site-specificity. Labeling analytes with a reporter protein (such as GFP, aequorin, luciferase, etc.) is often accomplished with techniques that link the analyte to certain amino acid residues via cross-linkers, such as NHS-ester-primary amino group (lysines) and maleimide-sulfhydryl group (cysteines) conjugation chemistries. The availability of a single amino acid (i.e. cysteine) in a reporter protein allows for the production of homogeneous conjugation products. Homogeneous conjugation products (a.k.a. homogeneously labeled analytes) are known to produce significantly better reproducibility in terms of their analytical signal, when compared with heterogeneously labeled analytes. Therefore, three of our five aequorin variants described herein are single cysteine moieties. None of the listed obelin variants contains a single cysteine, and this is due to the fact that previous work in our lab showed that cysteine-free and single cysteine obelin mutants showed very little bioluminescence activity, thereby limiting their utility as a label. Owing to this fact, we instead focused on the role of cysteine residues in obelin's bioluminescent response, in an effort to better understand what effect obelin's cysteines have on emission shifts and decay kinetics in that photoprotein.

Table II. Structure of the coelenterazine analogues utilized



Coelenterazine *e* does not have the C2 or C8 position altered, instead it has a two carbons added to the central amide group that fuse together the two rings. All remaining coelenterazine analogs have a C2 and/or a C8 substitution.

The importance of these cysteine residues in photoproteins has been indicated by the relatively high percentage of cysteines (along with His, Trp and Pro residues) in the structures of these proteins. It has been found that certain cysteine mutations in aequorin can cause significant reduction in the bioluminescent activity of these proteins, suggesting that they have an integral structural role (Kurose *et al*., 1989; Bondar *et al*., 1998, 2001). For this reason, as well as the superior analytical potential of single cysteine photoproteins for site-specific conjugation and immobilization, only

Table III. The emission maxima (nm) of all of the semi-synthetic photoprotein mutants

	ntv	*i*	*ip*	*cp*	*h*	*hcp*	*f*	*fcp*	*e*	*n*
WT-Aeq	471	491	456	459	473	456	490	470	473	473
Cys-free Aeq	479	491	453	456	479	455	488	466	479	482
Aeq S5C	474	490	458	456	477	456	487	470	479	481
Aeq E53C	480	492	453	458	479	453	490	467	476	481
Aeq M71C	477	498	467	453	477	458	484	479	478	485
WT-Obl	491	497	471	470	491	468	494	471	488	491
Obl C151S	476	493	471	471	476	470	488	473	474	479
Obl C158S	476	491	468	470	474	468	491	473	473	483
Obl C67+75S	491	497	471	471	491	471	494	473	488	494

The coelenterazine analog utilized is indicated in the first row, and the photoprotein mutant utilized is indicated in the first column. The reported spectra of all semi-synthetic photoprotein mutants are the average of triplicate spectra, which have been corrected for the blank.

cysteine residues were altered, for a total of four aequorin and three obelin mutants (Fig. 1). Obl C158S was chosen because of previous work by Bondar *et al*. (2001), which indicated the cysteine at position 158 was integral to high bioluminescence intensity in obelin, suggesting that it played an important structural role. We therefore wanted to explore whether or not this mutant would behave differently than WT-Obl when paired with coelenterazine analogues. Obelin was also mutated at position 151 and doubly mutated at positions 67+75 in order to examine the effect alternative cysteine mutations would have on obelins bioluminescent properties, and whether or not these mutants would behave in a manner similar to Obl C158S.

Shimomura *et al*. previously demonstrated that WT-Aeq will retain significant activity, while exhibiting shifted emission maxima and decay kinetics, when paired with various coelenterazine analogues containing altered substituents at the C2 and C8 positions (Shimomura *et al*., 1988, 1990, 1993; Inouye and Shimomura, 1997; Hirano *et al*., 1998; Zheng *et al*., 2000). For this reason, all seven of the photoprotein mutants, as well as WT-Aeq and WT-Obl, were paired with 10 such coelenterazine analogs (Table I). This cross-pairing produced a total of 70 semi-synthetic photoprotein mutants.

Next, we determined the bioluminescent properties, such as specific activity, emission maxima and decay half-life time, of the resulting semi-synthetic photoprotein mutants. The lack of any structural information on these aequorin and obelin mutants makes the interpretation of these data difficult. However, tentative conclusions can be drawn by assuming that the overall structures of these photoprotein mutants do not deviate greatly from their wild-type counterparts, a logical hypothesis since all mutants still exhibit bioluminescent activity. As can be observed in Table I most mutants exhibited a specific activity comparable with their wild-type counterparts. The bioluminescent activities of the bright Cys-free Aeq and Aeq E53C mutants offer the advantage of higher than wild-type specific activities. Also, even though a few of the mutants herein do show reduced activity when compared with wild-type, we believe that they still exhibit enough bioluminescence to qualify as useful analytical labels. We believe that the most significant loss of activity, in Obl C158S, can be explained by the fact that it is the only mutation located close to a calcium-binding EF hand loop, in this case EF hand 4. Therefore, this mutation may detrimentally interfere with the photoproteins ability to bind Ca^{2+}.

We obtained the bioluminescence emission maxima of the nine photoproteins paired with 10 different coelenterazine analogs by stimulating bioluminescence with a calcium triggering buffer and collecting the resulting emission spectra using a CCD camera (Table III). The functionality of the coelenterazine molecule caused similar shifts in the bioluminescence emission maxima of most of the aequorin and obelin mutants. In general, bulky substituents on the C2 group of coelenterazine led to red shifted emission maxima (coelenterazine *i*, *f*, *n*) whereas smaller non-aromatic substituents on the C8 group caused blue shifts in the emission spectra (coelenterazine *ip, cp, hcp*). These emission shifts would essentially cancel each other out when both a bulky C2 group and a small C8 group were present on the same

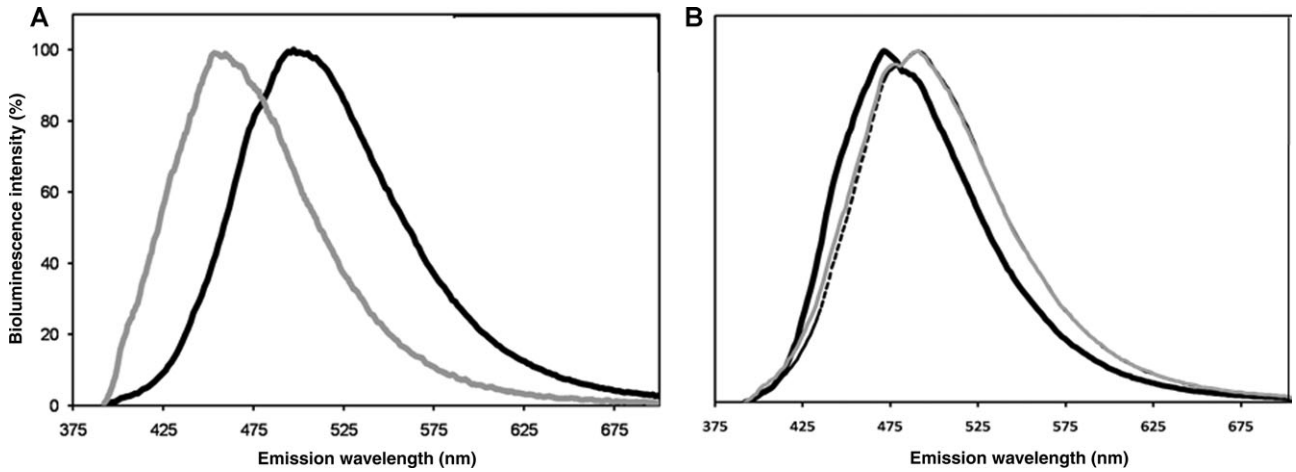


Fig. 2. (**A**) Sample emission spectra of Obl C67+75S with coelenterazine *i* (black line) and Cys-free Aeq with coelenterazine *ip* (gray line) showing the largest separation in the emission peaks of the semi-synthetic photoprotein mutants. (B) Sample emission spectra of WT-Aeq (black line), WT-Obl (dotted black line) and Obl C67+75S (gray line) with coelenterazine *ntv* showing the greatest separation in emission maxima among the mutants when incorporating the same coelenterazine. The reported spectra are the average of triplicate spectra, which have been corrected for the blank.

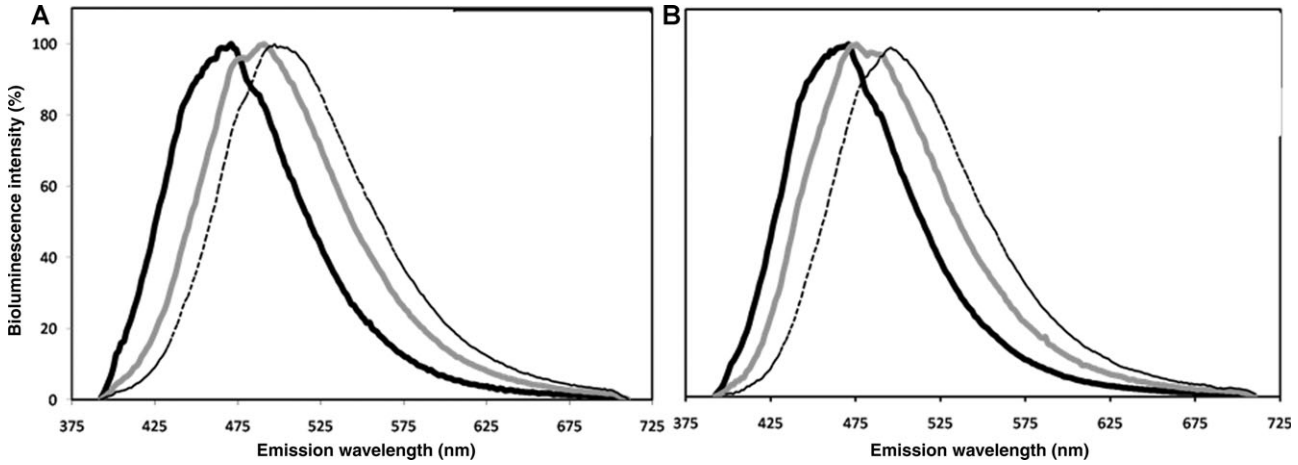


Fig. 3. (**A**) Emission spectra of Obl C67+75S with coelenterazine *cp* (black line), *ntv* (gray line) and *i* (dotted black line). (**B**) Emission spectra of Obl C151S with coelenterazines *cp* (black line), *ntv* (gray line) and *i* (dotted black line). The emission maxima of the obelin mutants shifted to varying degrees with these coelenterazines, depending on the cysteine mutations (WT-Obl and Obl C67+75S shifted in one manner and Obl C151S and Obl C158S shifted in a different manner). The reported spectra are the average of triplicate spectra, which have been corrected for the blank.

coelenterazine (coelenterazine *fcp*). The most important trend observed is that all seven of the photoprotein mutants, as well as the wild types, exhibited a large red shift in their emission spectra when paired with coelenterazine *i*, which has not been observed previously. Coelenterazine *i* contains iodine on the C2 phenyl group in place of a hydroxyl group. It is reasonable to assume that the presence of a halogen in both coelenterazine *i* and *f* combined with the retention of native C8 size and aromaticity is the cause of their large red shifts.

The various aequorin and obelin mutants also exhibited different spectral characteristics when paired with the same coelenterazine analog. For example, the emission maxima are different in WT-Obl and the double mutant Obl C67+75S when compared with Obl C151S and Obl C158S, when the same coelenterazine analog (*ip, cp, hcp, i, f)* was incorporated. The emission maxima blue shifted by ~20 nm in WT-Obl and the double mutant Obl C67+75S when coelenterazines *ip*, *cp* and *hcp* were utilized, but only a small blue shift (5 nm) was observed in Obl C151S and Obl C158S with the same coelenterazines (Fig. 3). Conversely, coelenterazines *i* and *f* red shifted the emission maxima by ~20 nm in Obl C151S and Obl C158S, whereas these coelenterazines effected only a minimal (6 and 4 nm) red-shift in WT-Obl and the double mutant Obl C67+75S (Fig. 3). Such spectral differences found among the mutants when incorporating the same coelenterazine analog are likely due to slight structural perturbations caused by the individual mutations and mutation combinations.

Table IV. The decay half-life time (s) of all of the semi-synthetic photoprotein mutants

	ntv	*i*	*ip*	*cp*	*h*	*hcp*	*f*	*fcp*	*e*	*n*
WT-Aeq	0.4	6.0	0.5	0.2	0.2	0.2	0.4	1.0	0.4	2.5
Cys-Free Aeq	0.6	17	0.6	0.3	0.8	0.5	0.6	0.5	0.6	1.9
Aeq S5C	0.6	24.1	0.7	1.0	0.3	0.7	0.8	0.3	0.7	1.7
Aeq E53C	0.8	20.9	0.8	0.2	0.7	0.5	0.7	0.5	0.6	1.9
Aeq M71C	0.6	12.7	0.5	0.2	0.4	0.2	0.9	0.3	0.7	1.5
WT-Obl	0.5	4.2	2.2	0.1	0.1	0.04	0.3	0.1	0.5	4.8
Obl C151S	0.5	7.1	1.0	0.2	0.2	0.2	0.4	0.2	0.7	2.1
Obl C158S	0.6	9.3	0.3	0.2	0.2	0.8	0.5	4.1	0.6	1.1
Obl C67+75S	1.8	17.6	8.9	0.4	0.2	0.7	0.8	0.3	1.9	10.9

The coelenterazine analog utilized is indicated in the first row, and the photoprotein mutant utilized is indicated in the first column. The reported decay half-life time is the average of triplicate measurements, which have been corrected for the blank.

The bioluminescence decay kinetics of all 70 of our semi-synthetic aequorin variants was then obtained by collecting the light emission over a 20 s time period following calcium stimulation. This decay curve was then fit with a one-phase exponential decay equation in order to determine the decay half-life time. The decay kinetics of the mutants also all exhibited similar trends with the various colenterazine analogs. In general, bulky substituents on the C2 group of coelenterazine caused longer decay half lives, and smaller, non-aromatic substituents on the C8 group caused shorter decay half lives (Table IV). It is important to note that no change in quantum yield was apparent in any of these photoprotein mutant-coelenterazine analog pairings. For example, although some coelenterazine analogs yielded decay half-life times of >20 s, whereas others flashed with a half-life of <0.5 s, the overall area under their decay curve did not change. In other words, as decay half-life time increased the maximal bioluminescence decreased proportionally. This is likely due to changes in the calcium sensitivity among our semi-synthetic photoprotein variants. This theory, where increased decay half-life times correspond to a decrease in calcium sensitivity and a decrease in maximal bioluminescence has been expounded upon in detail by Tricoire *et al*. (2006).

Coelenterazine *i* caused the most dramatic change in decay half-life times, ranging from 4.2 to 24.1 s, depending on the photoprotein mutant employed (Table IV). The length of this half-life essentially makes it short-lived glow-like in nature, unlike the traditional flash kinetics associated with both aequorin and obelin. Shimomura observed an analogously long half-life when pairing coelenterazine *i* with

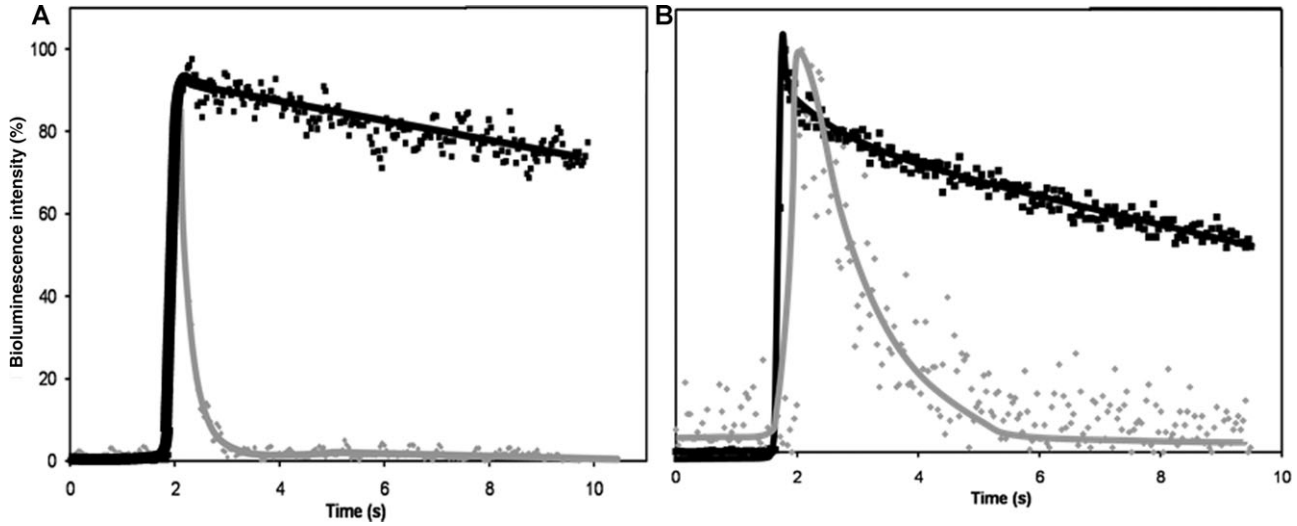


Fig. 4. (**A**) Sample graphs showing the significant difference in the decay kinetics of two photoprotein derivatives, Aeq M71C with coelenterazine *hcp* (gray line) ($t_{one\text{-}half} = 0.17$ s) and Aeq S5C with coelenterazine *i* (black line) ($t_{one\text{-}half} = 24.07$ s). (B) Sample graphs showing the difference in decay kinetics among two photoprotein mutants, Obl C158S (gray line) and Obl C67+75S (black line), with the same coelenterazine, coelenterazine *n*. The exhibited decay kinetics are the average of triplicate measurements, which have been corrected for the blank. The *y*-axis is normalized so that each semi-synthetic photoprotein mutant has a maximum signal of 100%: as in previous studies longer decay half-lives corresponded to decreased maximal bioluminescence intensity, indicating no change in overall quantum yield of the reaction.

WT-Aeq (8 s), as well as when pairing WT-Aeq with other coelenterazines containing different heavy atom functional groups (Shimomura *et al*., 1990). The coelenterazine *n* also resulted in extended decay half-life times in all of the mutants, although to a less extent than coelenterazine *i* (1.1–10.9 s). The commonality between these long half-life coelenterazines is their bulky C2 group. Toma *et al*. (2005) solved the crystal structure of WT-Aeq with both coelenterazine *i* and coelenterazine *n* and postulated that their extended decay half-lives were due to the larger C2 groups of coelenterazine *i* and coelenterazine *n* being inserted in between the helices of EF hand 3 and 4. This increased steric bulkiness interfered with the Ca^{2+}-induced conformational change, thereby decreasing the rate of the bioluminescence reaction and increasing the decay half-life time. It is reasonable to conclude that the increase in the decay half-lives of all our mutants with coelenterazine *i* and coelenterazine *n* is due to the same reason.

Coelenterazines *cp*, *h* and *hcp* exhibited significantly shorter decay half-lives when paired with the majority of the aequorin and obelin mutants. Coelenterazine *fcp* also exhibited short decay half-lives when paired with the obelin mutants, but not when paired with the aequorins. Coelenterazine *cp*, *hcp* and *fcp* all have a five-membered ring in place of the native phenyl at position C8, decreasing the size and eliminating the aromaticity of the C8 group. Structural analysis of WT-Aeq with coelenterazine *cp* suggested that its fast reaction kinetics was the result of the elimination of C8 hydrophobic interactions with the protein (Toma *et al*., 2005). The elimination of these interactions effectively 'frees' the EF hands to move faster following calcium binding, increasing the rate of the bioluminescence reaction and decreasing the decay half-life. This reasoning can also be applied to our mutants, with the short half-lives found in *cp*, *hcp* and *fcp* being explained by the lack of hydrophobic interaction capability in the C8 group.

The different mutations to the photoproteins also resulted in changes in the decay kinetics, when the same coelenterazine was incorporated. Most notably, the aequorin mutants behaved differently than the obelin mutants. For example, coelenerazine *h* gave shorter half-lives in all obelin mutants, but did not alter half-lives significantly in the aequorin mutants. These differences can likely be attributed to the slightly different three-dimensional structure of aequorin, when compared with obelin. Additionally, the double mutant, Obl C67+75S, exhibited significantly extended decay half-lives when compared with all other obelin mutants, and the majority of the aequorin mutants suggesting that cysteine residues C67 and C75 may play an important role in the calcium responsiveness of obelin.

Owing to their ultra-sensitive nature and genetic malleability, bioluminescent proteins are becoming more and more popular in fields, such as bioluminescence imaging, split reporter complementation and reconstitution methods, bioluminescence resonance energy transfer and miniaturized bio-analysis. These bioluminescence-based techniques have been used for the monitoring of important cellular events, such as protein–protein interactions, tumor progression, biomarker detection, etc. However, two of the major challenges when employing bioluminescent reporters in these methodologies, instead of fluorescent moieties, are the lack of spectral diversity for multiplexing and multimodal imaging and the difficulty in obtaining a strong enough light signal. The weak light emission problem is being largely solved by the advancement of sensitive detection devices, such as powerful confocal microscopes paired with the new electron coupling charge-coupled devices. The spectral diversity issue, however, still requires progress. Ideally, a wide range of bioluminescent colors should be available to the researcher, so that multiple cellular processes can be analyzed and imaged simultaneously. We believe that the spectral diversity and altered decay half-life times of the semi-synthetic photoprotein variants described herein will contribute significantly to enhancing the multiplexing capability of bioluminescent reporters. Moreover, the aequorin variants have the additional advantage of being either cysteine-free or single cysteine

moieties, which will allow for the facile site-specific immobilization and conjugation of these mutants. This advantage, combined with the altered bioluminescent characteristics of these photoproteins, lead us to believe that these new, semi-synthetic photoprotein mutants will increase both the breadth and the depth of applications that bioluminescent photoproteins are suitable for.

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