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Aequorin mutants with increased thermostability

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Abstract

Bioluminescent labels can be especially useful for *in vivo* and live animal studies due to the negligible bioluminescence background in cells and most animals, and the non-toxicity of bioluminescent reporter systems. Significant thermal stability of bioluminescent labels is essential, however, due to the longitudinal nature and physiological temperature conditions of many bioluminescent based studies. To improve the thermostability of the bioluminescent protein aequorin we employed random and rational mutagenesis strategies to create two thermostable double mutants, S32T/E156V and M36I/E146K, and a particularly thermostable quadruple mutant, S32T/E156V/Q168R/L170I. The double aequorin mutants, S32T/E156V and M36I/E146K, retained 4 and 2.75 times more of their initial bioluminescence activity than wild type aequorin during thermostability studies at 37 °C. Moreover, the quadruple aequorin mutant, S32T/E156V/Q168R/L170I, exhibited more thermostability at a variety of temperatures than either double mutant alone, producing the most thermostable aequorin mutant identified thus far.

Keywords

bioluminescent protein; aequorin; bioluminescence; mutagenesis; thermostable

Introduction

Aequorin is a bioluminescent photoprotein that has been widely employed in a variety of biological and analytical applications, including as an intracellular calcium indicator [1], in gene expression studies [2], drug discovery [3], and as a highly sensitive reporter in binding and immunological assays [4]. The bioluminescent signal of aequorin allows it to achieve extremely low detection limits in most biological fluids and matrices, due to the negligible native bioluminescent background signal in these mediums. This quality, along with its non-

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toxicity, gives bioluminescent labels, such as aequorin, an analytical advantage over fluorescent or colorimetric alternatives in certain types of studies [5].

However, bioluminescent proteins have undergone the extensive mutagenesis studies common to their fluorescent counterparts, and therefore have not been optimized in terms of their spectral diversity and physical characteristics. Of particular importance for both *in vivo* studies and versatile device development (such as assays and sensors) is the production of thermostable bioluminescent proteins [6]. The relatively high temperatures found in living cells and during point-of-care use require reporting proteins that can continue to function reliably when exposed to both ambient and physiological temperatures for extended periods.

Aequorin has two distinct units required for bioluminescence emission; the apoprotein (apoaequorin) and the chromophore, coelenterazine. Apoaequorin binds coelenterazine non-covalently within its hydrophobic core to form the functional photoprotein. Calcium can bind to the EF-hands of aequorin and cause a conformational change which results in the oxidation of the coelenterazine through an excited state, yielding coelenteramide, carbon dioxide, and the release of bioluminescent light ($\lambda_{em} = 470 \text{ nm}$) [7]. The bioluminescent signal, therefore, is triggered by the binding of calcium to a 'coelenterazine-charged' aequorin.

A variety of mutagenesis strategies have been undertaken with aequorin, both in order to understand its mechanism of bioluminescence and in an effort to manipulate its properties, most of which have been site-directed mutagenesis and substituted coelenterazine studies [8-20]. Random mutagenesis techniques however, have been used much less frequently, with only two publications thus far incorporating it into their strategies to improve upon the properties of native aequorin [9,10]. Random mutagenesis is a powerful tool that can be used to acquire protein mutants with improved and altered properties by rapidly screening large libraries of mutants. Random mutagenesis is especially useful for expanding the properties of a protein when the most obvious rational mutation sites have already been targeted. The cumulative effect of multiple mutations and/or mutations at non-essential sites is difficult to predict *ab initio*, but can often be identified using random mutagenesis screening methods. For these reasons, random mutagenesis has been used ubiquitously to successfully increase the thermostability of a variety of proteins [6, 21, 22].

In this study, we employed both the error-prone polymerase chain reaction (PCR) method of random mutagenesis and semi-rational mutation strategies in order to increase the thermostability of aequorin. A low mutation frequency error-prone PCR technique (0-2.6 mutations for aequorin) and colony screening for retained bioluminescent activity was first used in order to identify two double mutants that had an increased thermostability as compared to wild-type aequorin (S32T/E156V and M36I/E146K). Next, we combined the double mutant S32T/E156V with a previously discovered thermostable aequorin mutant, Q168R/L170I, in order to create a quadruple mutant, S32T/E156V/Q168R/L170I, which was more thermostable than any aequorin variant reported in the literature thus far. The emission spectra and decay half-life times of the relevant mutants paired with native and substituted coelenterazine analogues were also determined in order to identify the important spectral properties of these thermostable aequorin mutants.

Experimental

For an in-depth discussion of the materials and methods employed please refer to the Supplementary Information. In brief: random and site-directed mutagenesis reactions were performed using a plasmid containing the gene for wild type aequorin and ampicillin resistance. Randomly generated mutants were initially screened by determining the relative bioluminescence intensity of individual colonies, as compared to wild-type aequorin. Aequorin mutant colonies that exhibited significant bioluminescence were then expressed in *E. coli*, and the plasmid DNA was purified and sequenced for base identification and exact mutation site(s) determination. Next, the aequorin mutants were purified and characterized to determine their purity and concentration. Concentration of all of the mutants was normalized to that of wild-type aequorin, and the proteins were charged with identical molar excess amounts of coelenterazine. The thermostability of all mutants and wild-type aequorin was determined using two time course studies, one at room temperature for 2 months, and the other at 37 °C for six days. A heat shock study was also completed, in which the temperature varied from 25 to 60 °C. The most thermostable aequorin mutants, and wild-type aequorin, were also charged with multiple coelenterazine analogues, and their emission maximum and decay half-life time was determined using previously established methods (see Supplementary Information). Lastly, the mutants were analyzed with circular dichroism (Supplementary Information).

Results and Discussion

Bioluminescent properties of aequorin mutants

In order to produce thermostable aequorin mutants a low frequency error-prone PCR method was first employed on a wild-type aequorin template (WT aequorin). Approximately one thousand colonies were selected and tested for the retention of bioluminescence activity. Fourteen colonies exhibited significant bioluminescence activity, ranging from 4% to 60% of the activity of WT aequorin. Six of the single mutants from random mutagenesis, D114E, K30R, E84D, H27R, E156D, and A81S, all had decreased, but still substantial, bioluminescence activity (Table 1, Column 2). These single mutants had slightly altered bioluminescence emission maxima and decay half-life times (Table 1, Column 3 and 4). The five double mutants, H18Q/N12D, K96R/I139V, S32T/E156V, M36I/E146K, and N46K/K134R retained 60%-15% of the WT activity, whilst the triple mutant N46K/M71V/D92E had only 10% of its original activity remaining (Table 1, Column 2).

Following the initial random mutagenesis study the most thermostable aequorin mutant identified, S32T/E156V, was combined with a previously discovered thermostable aequorin mutant, Q168R/L170I to form the quadruple mutant, S32T/E156V/Q168R/L170I. To study synergistic effects of the multiple mutations in this quadruple mutant the single mutants S32T and E156V were also created, and compared to both WT aequorin and a cysteine free (Cys->Ser) aequorin mutant, Mut S [23]. The resulting single mutants retained between 45-88% the original bioluminescent activity of WT aequorin (Table 1, Column 2). The emission maxima of any mutants containing the Q168T/L170I combination exhibited an approximately 5 nm blue-shift (Table 1, Column 3). This shift is possibly due to the presence of these amino acids in the coelenterazine reaction core of aequorin and their

proximity to residue His169, which is part of an integral coelenterazine stabilizing triad and believed to be essential to the oxidation of coelenterazine to coelenteramide upon calcium binding [24].

Lastly, the mutants created were paired with two additional coelenterazine analogues other than native coelenterazine (ctz *ntv*); coelenterazine *i* (ctz *i*) and coelenterazine *hcp* (ctz *hcp*). The emission maximum and decay half-life times of these ctz *i* or ctz *hcp* paired aequorin mutants was then determined (Table 1, Column 3 and 4). Previous studies have shown that ctz *i* yields both a red-shifted emission maximum and an extended decay half-life time, whilst ctz *hcp* incorporation results in a blue-shifted emission maxima and a shortened decay half-life time in both WT and various aequorin mutants [17, 19, 20]. These previous trends remained with all of our current mutants (Table 1, Column 3 and 4). The altered emission maxima and decay half-life times imparted by these alternative coelenterazine analogues should increase the potential multi-analyte detection capability of the thermostable mutants identified herein, both by spectral and temporal resolution.

Thermostability of aequorin mutants

Following the initial random mutagenesis study only two double mutants yielded a significant increase of thermostability, S32T/E156V and M36I/E146K. These two mutants retained 32% and 22% of their original bioluminescence activity, following a 72 h incubation at 37 °C, as compared to an 8% retention in WT aequorin (Table 1, Column 5). This increase in thermostability may be a result of subtle changes in the conformation of aequorin due to the simultaneous mutations, since both Met36 and Glu146 are located in the middle of helices that compose calcium binding EF-hands (see Figure S1 in the Electronic Supplementary Information), and do not contribute directly to coelenterazine or calcium binding [7], but Ser 32 and Glu 156 occur either at the very beginning, or within of the calcium binding loops of the EF-hands.

The single mutant S32T retained 13% of its bioluminescence activity when incubated at 37 °C for 72 h, whereas the single mutant E156V retained 26% (Table 1, Column 5). This indicates that the two mutations combine to form an increased thermostability through cumulative effects in the S32T/E156V double mutant (32% of original activity during 37 °C, 72 h study), with the S32T mutation being more important for the increase in thermostability. We postulated that combining these S32T/E156V mutations in aequorin with a previously identified thermostable aequorin mutant, Q168R/L170I, may yield a mutant more thermostable than either of the double mutants alone [9, 10]. Indeed, this was our result when we constructed and analyzed the quadruple mutant, S32T/E156V/Q168R/L170I. The mutant S32T/E156V/Q168R/L170I retained 75% of its original bioluminescence activity after a 72 h incubation at 37 °C, which is significantly greater than the 32% and 61% retained by the double mutants S32T/E156V and Q168R/L170I under identical conditions (Table 1, Column 5). Additionally, we extended the 37 °C thermostability study for 6 days (144 h) with our quadruple mutant and S32T/E156V/Q168R/L170I retained approximately 50% of its original activity even after 6 days at 37 °C. This was a significantly greater bioluminescence activity retention than any other mutant or control studied, including the most thermostable aequorin mutant previously identified in the

literature, Q168I/L170I, which retained only 30% of its activity after 6 days (Figure 1) [9,10].

A thermal inactivation study at room temperature, conducted over 60 days, was also undertaken with the aforementioned aequorin mutants, with the quadruple mutant, S32T/E156V/Q168I/L170I, retaining the most bioluminescence activity at the end of 60 days (Electronic Supplementary Information, Figure S2). This result corresponds to the heat shock experiments that were undertaken by incubating the two thermostable mutants (S32T/E156V/Q168I/L170I and Q168R/L170I) and two control aequorins (WT and Mut S) at temperatures varying between 25 °C and 70 °C for 30 min and measuring remaining bioluminescence (Figure 1). In this experiment, a drastic difference in short-term thermostability between the mutant aequorins and WT aequorin was not noticed until a temperature of 40°C was reached, at which point the thermostability diverged greatly until 70 °C was reached. At this point (70 °C) all variants precipitously lost activity (Figure 1). The increase in thermostability in mutant Q168R/L170I may be due to a stabilization of the local aequorin structure surrounding His169, which is a required component for the oxidation of coelenterazine to coelenteramide during the bioluminescence reaction of aequorin [9]. This stabilization combined in a synergistic way with our S32T/E156V mutations in order to cause a significant increase in the thermostability of the quadruple mutant, which was greater than the thermostability of either double mutant alone.

Conclusions

The quadruple aequorin mutant designed in this study, S32T/E156V/Q168I/L170I, was significantly more thermostable at a variety of temperatures than any other aequorin mutant reported to date. However, the extent of its increase in thermal tolerance was decidedly more pronounced at physiological temperatures over a 3-6 day time period (37 °C). This fifteen to twenty percent increase in thermostability over any currently reported aequorin mutants may enhance aequorins utility for *in cellulo* studies, considering the physiological temperature and often longitudinal nature of such studies. Moreover, our quadruple mutant showed increased stability at room temperature, improving aequorins adaptability to point of care and portable analytical devices, since these instruments must often be stored at ambient temperatures. The thermostable aequorin mutant also exhibited altered emission maxima and half-lives when paired with substituted coelenterazine analogues. These varied colors and decay kinetics could potentially facilitate multiplexing bioluminescence detection and multi-modal imaging. Future work in this area should concentrate on further increasing both the thermostability and the bioluminescence activity of aequorin via additional rounds of random and/or rational mutagenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Graph A showing thermal inactivation study results of WT, Mut S, Q168R/L170I, and S32T/E156/Q168R/L170I aequorins with their remaining bioluminescence activity (y axis) plotted against the time incubated at 37 °C (x axis) over a 6 day time period. Graph B showing heat shock study results of WT, Mut S, Q168R/L170I, and S32T/E156/Q168R/L170I aequorins with their remaining bioluminescence activity (y axis) plotted against the temperature (x axis).

Table 1

Total bioluminescence activity of all mutants identified, emission maxima of bioluminescence when paired with coelenterazine *ntv*, *i*, and *hcp*, and bioluminescence decay half-life time of all mutants when paired with coelenterazine *ntv*, *i*, and *hcp*. The last column shows the bioluminescence activity remaining for each individual mutant after being incubated at 37 °C for 72 h. The bioluminescence activity at time zero was considered 100% for each individual mutant in the (Activity 72 h) column, whereas the activity reported in the (Activity) column is the percentage of bioluminescent activity of the mutant when compared to WT aequorin.

Mutation	Activity (%)	$\lambda_{\max}(\text{nm})$ <i>ntv (i,hcp)</i>	$t_{1/2}(\text{s})$ <i>ntv (i,hcp)</i>	Activity 72 h (%)
WT	100	475(488,460)	0.48(7.04,0.28)	8
Mut S	116	472(489,454)	0.68(16.35,0.14)	-
S32T	88	474(488,454)	0.52(5.34,0.25)	13
Q168R/L170I	80	468(485,449)	0.78(9.48,0.19)	61
E156V	76	474(489,454)	0.61(6.75,0.30)	26
H18Q/N121D	60	478(489,460)	0.56(6.64,0.25)	-
A81S	58	475(488,458)	0.49(7.87,0.24)	4
K96R/I139V	55	477(488,460)	0.51(5.61,0.24)	2
S32T/E156V	53	474(488,458)	0.36(3.97,0.23)	32
E156D	53	475(488,458)	0.46(5.85,0.25)	3
H27R	53	477(488,460)	0.49(5.94,0.24)	7
E84D	50	477(488,460)	0.46(7.21,0.27)	1
S32T/E156V/ Q168R/L170I	45	469 (485,449)	0.68 (8.92,0.23)	75
M361I/E146K	41	474 (485,455)	1.27 (29.66,0.22)	22
K30R	41	477 (488,460)	0.47 (6.59,0.25)	6
D114E	30	477 (488,460)	0.46 (6.13,0.23)	3
H58Y	27	485 (494,472)	1.74 (11.07,0.23)	-
N46K/K134R	15	477 (486,460)	0.29 (3.59,0.23)	-
N46K/M71V/D92E	10	475 (488,458)	0.32 (3.75,0.23)	-
133A	4	475 (489,457)	0.90 (10.55,0.24)	1