**INTRODUCTION**

Renilla luciferase (RLuc) is commonly used as a reporter gene either on its own or in conjuction with firefly luciferase. Its use in bioluminescence imaging, however, has been hampered by the blue-peaked emission spectrum generated when the enzyme oxidizes its substrate coelenterazine, as blue wavelength photons are strongly attenuated in biological tissues. To overcome this difficulty, we have explored red-shifting the emission spectrum of RLuc to increase its utility for small animal imaging applications. As shown below, the reaction catalyzed by RLuc involves a dioxygen intermediate, and the energy released by the break-down of this bond leaves the reaction product coelenteramide in an excited state. The excited electron/coelenteramide system can lose energy and return to the ground electronic state through a number of processes, with the desired transition in bioluminescence resulting in a photon of light. The wavelength of this photon depends on the energy difference between the excited and ground states, which in turn depends on the local chemical environment the coelenteramide finds itself in. The phenolate anion of coelenteramide is shown below as the emitting species, although assignment of the exact state of the emitting species is a matter of contention.

It has been demonstrated that various chemical environments will lead to different fluorescence emission peaks from coelenteramide; the species thought to be responsible for these distinct emissions are shown below. Although a range of emission shifts were created with the single residue mutants, these alterations in the putative active pocket invariably led to significant reductions in enzymatic activity. In the hopes of recovering some of this loss, an initial round of random mutagenesis was done on the F261W and F262W mutants with screening of around $1 \times 10^5$ clones. For rapid screening, these clones were initially selected for brightness and color by airbrushing on substrate and imaging using an IVIS-200 (Xenogen). Protein from promising clones was then purified and subjected to further testing. The information garnered from this initial random mutagenesis screen was utilized for planning a sequence of saturation mutagenesis studies at particular residue pairs on RLuc as well as the F261W and F262W mutants. Following several rounds of evolution, the most promising variants were:

**HYOMOLOGY MODEL/PUTATIVE ACTIVE SITE**

Renilla luciferase shows high homology (34-56% similar) to a number of bacterial haloalkane dehalogenases. This allowed generation of a homology model of RLuc using SWISS-MODEL (v3.5) and crystal structures of the haloalkane dehalogenase LinB from *Spingomonas paucimobilis* (PDB files 1i2k, 1k63, 1k6e, 1iz7, and 1m5j). The resultant model is shown below, with the N-terminal in blue and the C-terminal in red. Based on data from the dehalogenases, the putative active site was identified and the orientation of coelenterazine in the site was estimated. In addition to the estimated substrate location, the catalytic triad of D120, E144, and H225 is shown as well. The neighboring schematic highlights information, derived from the homology model, as to which active site residues likely interact with the substrate and were targets for site-directed mutagenesis.

**ENZYMATIC POCKET MUTATIONS**

Site-directed mutagenesis of the selected residues was performed on the previously described plasmid pBAD-pefl-RLuc [9]. This plasmid allows for periplasmic expression due to the pelB leader sequence, as well as nickel affinity purification using an encoded 6xHis tag. Activity of purified protein was measured with a 10 second integration following addition of 0.5 μg coelenterazine to dilute protein in 100 μl 100 mM Na-phosphate buffer (pH 7), with a correction applied for the spectral sensitivity of the luminometer. Emission spectra at ambient temperature were measured using a Triax 320. A total of 74 site specific mutations at 22 residues were created and analyzed, the more interesting mutations are presented below.

**PROTEIN EVOLUTION FOR A GREEN RLuc**

Although a range of emission shifts were created with the single residue mutants, these alterations in the putative active pocket invariably led to significant reductions in enzymatic activity. In the hopes of recovering some of this loss, an initial round of random mutagenesis was done on the F261W and F262W mutants with screening of around $1 \times 10^5$ clones. For rapid screening, these clones were initially selected for brightness and color by airbrushing on substrate and imaging using an IVIS-200 (Xenogen). Protein from promising clones was then purified and subjected to further testing. The information garnered from this initial random mutagenesis screen was utilized for planning a sequence of saturation mutagenesis studies at particular residue pairs on RLuc as well as the F261W and F262W mutants. Following several rounds of evolution, the most promising variants were:

**IN VITRO EVALUATION**

To demonstrate the applicability of these generated RLuc variants as mammalian reporter genes, expression vectors were constructed for RLuc, RLuc8, and RLuc8.6-535 in a pcDNA 3.1 backbone under the control of a constitutive promoter (CMV). 48 hrs following transfection of these plasmids into 293T cells, cycloheximide was added to allow assessment of the intracellular stability of the proteins. The results, shown below, indicate that RLuc8.6-535 is significantly brighter than RLuc and RLuc8, and has a similar intracellular half-life to RLUC8.

**IN VIVO EXPECTATIONS**

Theoretical estimates were made as to the benefit these red-shifted RLuc variants could be expected to show in vivo, by calculating the expected percent of photons that would be transmitted through 0.1 cm or 0.5 cm of rat liver tissue [9]. An additional parameter was calculated that took into account the variant’s specific activity as well as its emission spectrum. This parameter, “effective output”, shows the relative benefit of each variant versus native Renilla luciferase.

**CONCLUSION**

- Mutagenesis of RLuc8 led to emission-shifted variants of the enzyme, with spectra shifts up to ~60 nm. The green-emitting luciferases presumably favor the pyrazine anion resonance form of coelenteramide in their enzymatic pocket.
- Mutations at D126 were critical for generating large red-shifts. This residue is immediately adjacent to the putative active site residue I163.
- Of the developed variants, RLuc8.6-535 is expected to be the best for in vivo, as it is 6-fold brighter than native Renilla luciferase and emits light with a mean wavelength of 550 nm. The green-peaked emission spectra should allow significantly more photons to escape through biological tissue.

**REFERENCES**


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