Alignment Guided Mutagenesis of *Renilla* Luciferase Increases Stability and Light Output

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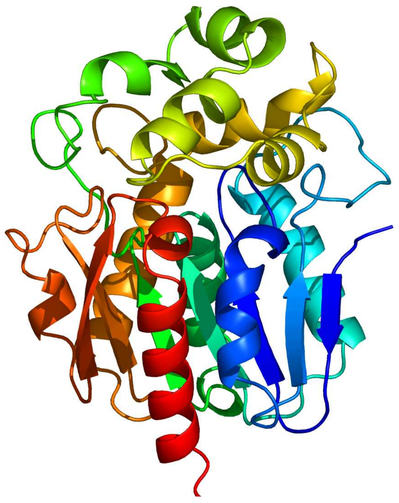
INTRODUCTION

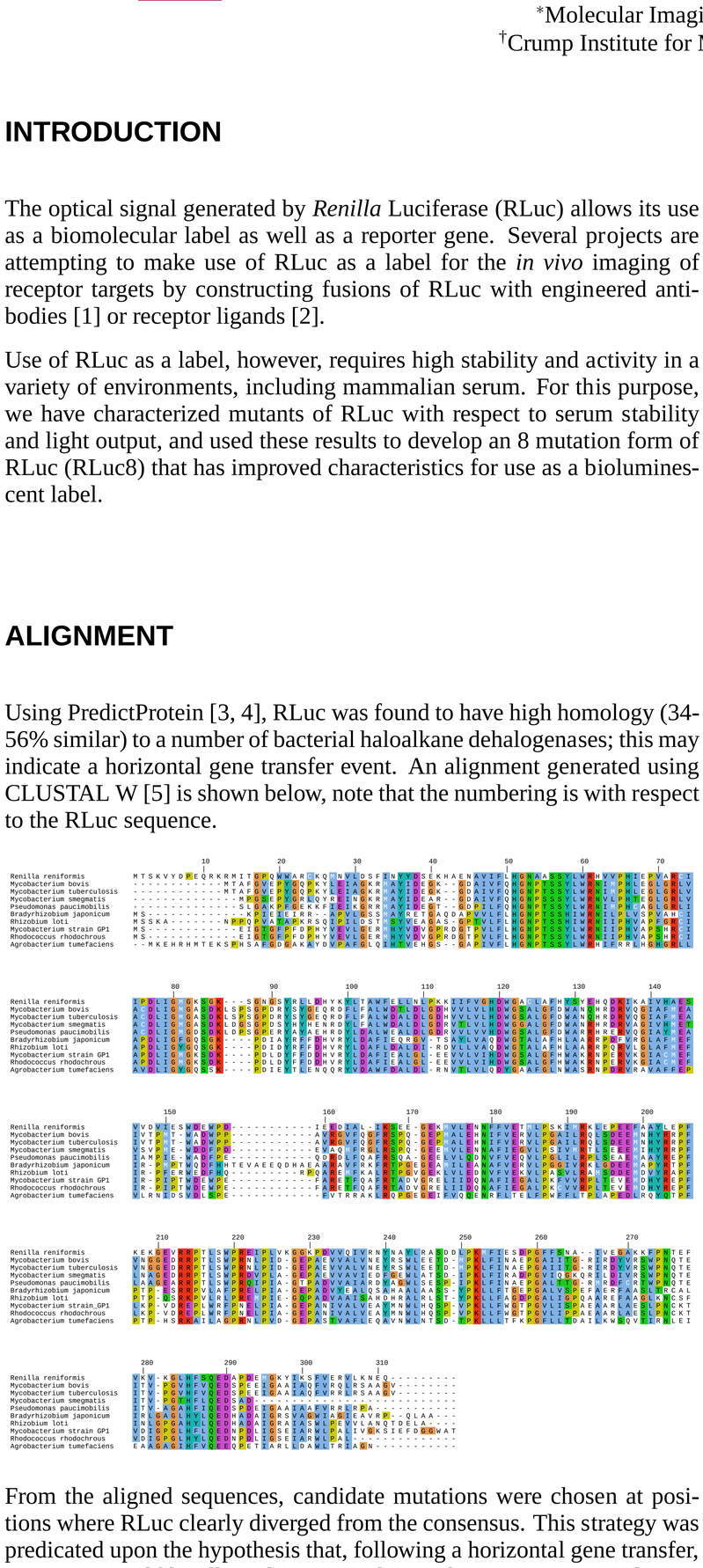
The optical signal generated by *Renilla* Luciferase (RLuc) allows its use as a biomolecular label as well as a reporter gene. Several projects are attempting to make use of RLuc as a label for the *in vivo* imaging of receptor targets by constructing fusions of RLuc with engineered antibodies [1] or receptor ligands [2].

Use of RLuc as a label, however, requires high stability and activity in a variety of environments, including mammalian serum. For this purpose, we have characterized mutants of RLuc with respect to serum stability and light output, and used these results to develop an 8 mutation form of RLuc (RLuc8) that has improved characteristics for use as a bioluminescent label.



ALIGNMENT

Using PredictProtein [3, 4], RLuc was found to have high homology (34-56% similar) to a number of bacterial haloalkane dehalogenases; this may indicate a horizontal gene transfer event. An alignment generated using CLUSTAL W [5] is shown below, note that the numbering is with respect to the RLuc sequence.



From the aligned sequences, candidate mutations were chosen at positions where RLuc clearly diverged from the consensus. This strategy was predicated upon the hypothesis that, following a horizontal gene transfer, mutations would be allowed to accumulate in the RLuc sequence that are neutral in the context of *Renilla* cytoplasm but could be deleterious in the more varied environments that a bacterial enzyme encounters.

Previous work in the literature [6] has shown that a Cys124Ala mutation (Cys152Ala in [6]) stabilizes RLuc, and that Cys73 (Cys101 in [6]) is required for activity. In light of the alignment data, Cys73 can be seen to be absolutely conserved across the enzymes, while C124 differs from the bacteria consensus. Based on the known required residues for haloalkane dehalogenases, Asp120, Glu144, and His285 are likely critical for RLuc activity as well.

HOMOLOGY MODEL

A homology model of RLuc was constructed so that the location of mutational candidates could be identified. This model was built using SWISS-MODEL (v3.5) [7] and crystal structures of the haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* (PDB files 1iz8, 1k63, 1k6e, 1iz7, and 1mj5). In the model below, the N-terminus is blue and the C-terminus is red. Based on data from the dehalogenases, the active site of RLuc is most likely at the intersection of the red, orange, and yellow helices.

METHODS

Mutagenesis

A human codon optimized version of rluc (Promega) was cloned into the pBAD expression system (Invitrogen) which adds a Myc epitope and a 6xHis tag to the C-terminus of the construct. As part of this cloning, a pelB leader sequence was added to the N-terminus of RLuc in order to direct expression into the bacterial periplasm. The C124A mutation was then introduced using a site-directed mutagenesis kit (Stratagene). All additional mutations were done on top of the C124A mutation.

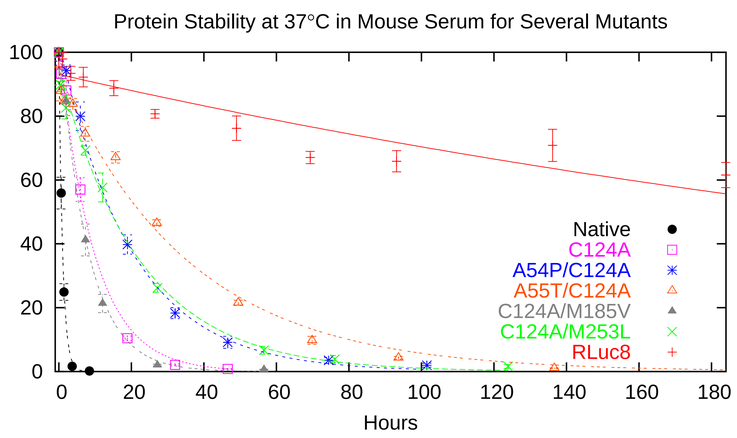
Protein Production/Purification

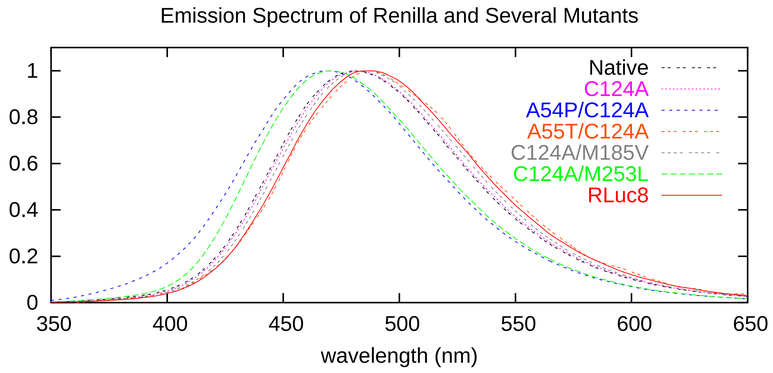
All proteins were produced in *E. coli* LMG 194 cells grown at 32°C. Cultures were allowed to come to an OD_{600} of 0.7, and arabinose was added to a final concentration of 0.2%. 12-14 hours later, cells were harvested and the periplasm extracted by osmotic shock [8]. The His-tagged protein was then purified by nickel affinity chromatography (Ni-NTA Superflow, Qiagen), with the elution buffer consisting of 300 mM NaCl, 20 mM HEPES, 250 mM Imidazole, pH 8. After determination of protein concentration, human serum albumin was added to 1% as a carrier protein, and the samples were stored at 4°C.

Testing

Protein concentration measurements were made using the Bradford assay with human serum albumin as the standard. Luciferase activity was measured by adding 1 μl of sample (diluted as necessary) to 100 μl room temperature 100 mM sodium phosphate buffer (pH 7), adding 0.5 μg coelenterazine, and reading for 10 seconds in a Turner 20/20 luminometer. Serum stability measurements were done by mixing 0.5 μl dilute luciferase with either 20 μl mouse serum or 50 μl rat serum, incubating at 37°C, and removing aliquots for activity testing. Serum half-lives were determined by least-squares fitting of mono-exponentials to the activity curves. Emission spectra at ambient temperature were measured using a Triax 320 (Jobin Yvon Horiba), which incorporates an optical grating device with a liquid N_2 cooled CCD detector.

RESULTS





Mutation	Specific Activity (RLU/pg)	Serum $\tau_{1/2}$ (hrs) mouse	rat	Wavelength (nm) peak	mean
Native	41	0.7	0.5	482	497
C124A	54±3	7.1±0.4	6.6±0.5	482	498
F33R/I34M/C124A	5	0.3	0.3	481	497
E44G/C124A	38	2.6	3.3	486	502
A54G/A55G/C124A	4	2.4	3.0	476	492
A54P/A55T/C124A	7	119	129	470	483
A54P/C124A	2	14	13	468	482
A55T/C124A	80	30	29	486	504
F116L/C124A	9	11	9.4	486	502
C124A/S130A	66	18	14	482	498
C124A/K136R	124±18	12	11	482	498
C124A/A143M	85	30	29	480	497
C124A/F180A	1	1.6	1.6	488	504
C124A/M185V	148	5.7	3.7	485	500
C124A/M191L	48	6.5	5.1	480	496
C124A/E195S/P196D	5	1.0	0.7	482	498
C124A/F199M	25	6.7	6.0	480	495
C124A/L203R	17	2.7	2.2	484	501
C124A/G229E	1	1.9	1.8	473	490
C124A/Q235A	46	3.3	3.6	473	489
C124A/M253L	88	15	10	471	488
C124A/S257G	42	1.3	1.4	477	493
C124A/F261L/F262L	0		N/A		N/A
C124A/F262L	2	5.8	6.4	478	495
C124A/S287L	168	28	20	478	496
C124A/M295I	49	5.0	4.9	480	497
C124A/K300A	31	3.5	3.9	481	497

The 7 mutations that had the most favorable increases in specific activity and/or stability, along with not leading to significant shifts in the emission spectra, were combined along with the C124A mutation into a single protein (RLuc8) The 8 mutations present in RLuc8 are A55T, C124A, S130A, K136R, A143M, M185V, M253L, and S287L. Note that during the cloning of RLuc8, the Myc epitope was removed from the construct. In order to facilitate a valid comparison, a C124A mutant construct was also created without the Myc epitope.

Mutation	Specific Activity (RLU/pg)	Serum $\tau_{1/2}$ (hrs) mouse	rat	Wavelength (nm) peak	mean
C124A ΔMyc	58±2	4.0	4.5	481	499
RLuc8	200±8	197	108	487	503

CONCLUSION

The combination of 8 favorable mutations generated a mutant *Renilla* luciferase (RLuc8) that exhibited a greater than 150-fold stability improvement in murine serum when compared to RLuc (<1 hr versus >100 hr). In addition to being more stable, RLuc8 also exhibited at least a 4-fold improvement in light output, along with a 5 nm emission spectrum red shift with respect to the native luciferase. This optimized *Renilla* luciferase represents a significant improvement that will allow its use for the labeling of biomolecules and following these constructs *in vivo*.

References

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