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 [3] or receptor ligands [4].

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 holoalkane dehydrogenases; 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 [3] has shown that a Cys124Ala mutation (Cys152Ala in [4]) stabilizes RLuc, and that Cys73 (Cys101 in [4]) 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 holoalkane dehydrogenases, 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) [5] and crystal structures of the holoalkane dehydrogenase LinB from Sphingomonas paucimobilis (PDB files 1x28, 1x63, 1x6c, 1x7, and 1m5). In the model below, the N-terminus is red and the C-terminus is blue. Based on data from the dehydrogenases, 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 fluc (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 pEB 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 OD600 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 [3]. 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 at 10 seconds in a Turner 20/20 luminometer.

RESULTS

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 A5ST, C124A, S130A, K136R, A143M, M185V, M235L, 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.

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 >10 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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Table 1: Specific Activity (RLuc/ppm) and Wavelength (nm) for Various Mutations

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Specific Activity</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>200±18</td>
<td>487±50</td>
</tr>
<tr>
<td>A5ST/C124A</td>
<td>197±10</td>
<td></td>
</tr>
<tr>
<td>A5ST/C124A/M185V</td>
<td>197±10</td>
<td></td>
</tr>
<tr>
<td>A5ST/C124A/M185V/M235L</td>
<td>197±10</td>
<td></td>
</tr>
<tr>
<td>RLuc8</td>
<td>200±18</td>
<td>487±50</td>
</tr>
</tbody>
</table>

Figure 1: Protein Stability at 37°C in Mouse Serum for Several Mutations