Bioluminescence Hot Paper

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Firefly Luciferase Mutants Allow Substrate-Selective Bioluminescence Imaging in the Mouse Brain

*Spencer T. Adams, Jr., David M. Mofford, G. S. Kiran Kumar Reddy, and Stephen C. Miller**

Abstract: *Bioluminescence imaging is a powerful approach for visualizing specific events occurring inside live mice. Animals can be made to glow in response to the expression of a gene, the activity of an enzyme, or the growth of a tumor. But bioluminescence requires the interaction of a luciferase enzyme with a small-molecule luciferin, and its scope has been limited by the mere handful of natural combinations. Herein, we show that mutants of firefly luciferase can discriminate between natural and synthetic substrates in the brains of live mice. When using adeno-associated viral (AAV) vectors to express luciferases in the brain, we found that mutant luciferases that are inactive or weakly active with* D*-luciferin can light up brightly when treated with the aminoluciferins CycLuc1 and CycLuc2 or their respective FAAH-sensitive luciferin amides. Further development of selective luciferases promises to expand the power of bioluminescence and allow multiple events to be imaged in the same live animal.*

Mice do not ordinarily glow in the dark. But if you introduce the enzyme firefly luciferase and the small molecule D-luciferin, they will glow in much the same way as a firefly does. Detection of the light emitted from these animals can be used to spy on specific events that would otherwise be invisible, such as gene expression and enzyme activity.[1,2] One limitation of this powerful technique has been the paucity of distinguishable luciferins and luciferases, which restricts our ability to visualize multiple events in the same live animal by using bioluminescence.

Firefly luciferase is particularly well suited to in vivo imaging.[2] The wavelength of the emitted light extends well into the red and near-infrared wavelengths that pass most easily through living tissues. Furthermore, the luciferin substrate is stable, easily administered, and offers several strategies to create luminescent probes.[2–5] In efforts to expand upon the bioluminescent palette of the firefly while retaining its inherent benefits, we have synthesized new luciferin analogues and mutated the luciferase enzyme.[6–8] We found that mutant luciferases can discriminate between D-luciferin and aminoluciferin analogues in vitro and in live cells.[7,8] However, part of the basis for this discrimination is a loss of substrate affinity, and it has been unclear how this discrimination would manifest in the context of live animals.[9]

Herein, we present bioluminescence imaging in the brain, and show that in this challenging environment, firefly luciferase mutants can discriminate between the natural substrate D-luciferin and synthetic luciferin analogues. We find that a balance of substrate selectivity and substrate affinity must be achieved to maintain high photon flux in vivo, where more restrictive conditions exist than in vitro. Nonetheless, simple point mutants of luciferase are capable of discriminating between substrates. This work will help guide the development of additional luciferase/luciferin pairs that can be used to shed light on the molecular basis of disease and drug action in live animals.

We recently reported that the combination of three active-site mutations (R218K, L286M, and S347A) yielded a luciferase with 10,000-fold selectivity for a synthetic aminoluciferin substrate over the natural substrate D-luciferin (Figure 1).[8] However, this selectivity came at the cost of an increased apparent Km compared to the individual mutants and the wild-type luciferase. A higher Km is not an issue for bioluminescence assays performed in vitro, but it could pose a problem for in vivo imaging, where substrate access is limiting.[9]

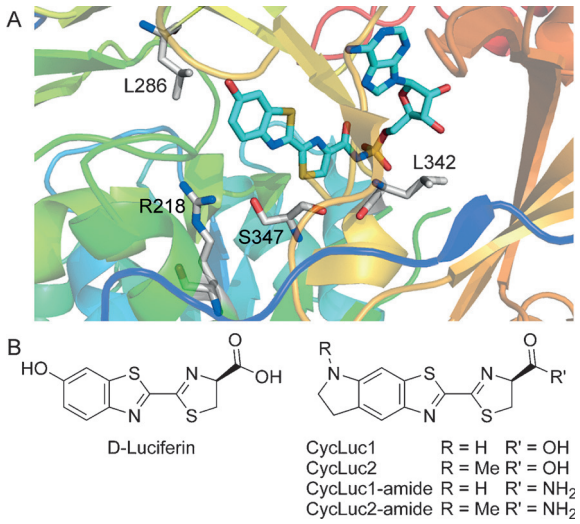

Figure 1. A) Crystal structure of firefly luciferase bound to a luciferyl adenylate analogue[10] (PDB ID: 4G36, rendered in PyMOL). Mutations to R218, L286, and S347 in the luciferin binding pocket have allowed tuning of the substrate specificity.[7,8] L342 does not contact the core luciferin structure and is distal to the structural differences between D-luciferin, CycLuc1, and CycLuc2 (B).

[*] S. T. Adams Jr., Dr. D. M. Mofford, Dr. G. S. K. K. Reddy, Prof. S. C. Miller
Department of Biochemistry and Molecular Pharmacology
University of Massachusetts Medical School
364 Plantation St Worcester, MA 01605 (USA)
E-mail: Stephen.miller@umassmed.edu
Homepage: http://www.umassmed.edu/bmp/faculty/miller/

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Light emission from a luciferin substrate requires activation by ATP to form an adenylated intermediate (Figure 1 and Figure S1 in the Supporting Information). Although residues in the ATP binding site make no direct contact with the core luciferin scaffold, the ATP binding site can influence (and be influenced by) luciferin binding. High ATP concentrations exacerbate the product inhibition that is observed with aminoluciferins (Figure S2). The apparent Km value for ATP depends on the luciferin substrate and is much lower for the aminoluciferins CycLuc1 and CycLuc2 compared to D-luciferin (Table 1, Figure S2). Taking advantage of this behavior can thus conceivably be used to distinguish between luciferins. Mutation of leucine 342, part of the GYGLTE motif found in many adenylate-forming superfamily members,[11] can greatly increase the apparent Km values for both D-luciferin and ATP and yet retain high maximal luciferase activity.[12] We found that the L342A mutation confers substrate selectivity that compares favorably to that of the previously reported R218K mutant (Figure 2 and Figures S2–S3). The change in apparent Km values for aminoluciferins is muted compared to D-luciferin (Table 1). Combination of the two mutations into a single luciferase results in further selectivity over D-luciferin (Table 1, Figure 2). Although not as selective as the R218K/L286M/S347A luciferase,[8] the R218K/L342A double mutant yields the highest maximal sustained photon flux with a synthetic luciferin to date, and no longer suffers from product inhibition (Figure 2, Figures S2–S3).

Table 1: Apparent Km values for luciferins and ATP with wild-type and mutant luciferases. NA = poor fit of data to a sigmoidal dose–response curve.

$K_{m(app)}$ [μM]	D-Luciferin Luciferin	ATP	CycLuc1 Luciferin	ATP	CycLuc2 Luciferin	ATP
WT[a]	6.2 ± 0.18	68 ± 3.0	NA	NA	0.69 ± 0.08	NA
R218K[a]	157 ± 7.7	408 ± 19	2.0 ± 0.22	50 ± 5.3	NA	16 ± 3.0
L342A[a]	232 ± 13	1320 ± 52	4.2 ± 0.39	96 ± 15	0.81 ± 0.06	22 ± 2.3
R218K + L342A[b]	> 250	> 4000	100 ± 4.1	398 ± 51	40 ± 2.1	238 ± 17

[a] ATP titrations were performed at 100 μM D-luciferin, 10 μM CycLuc1/2. [b] ATP titrations were performed at 1 mM D-luciferin, 100 μM CycLuc1/2.

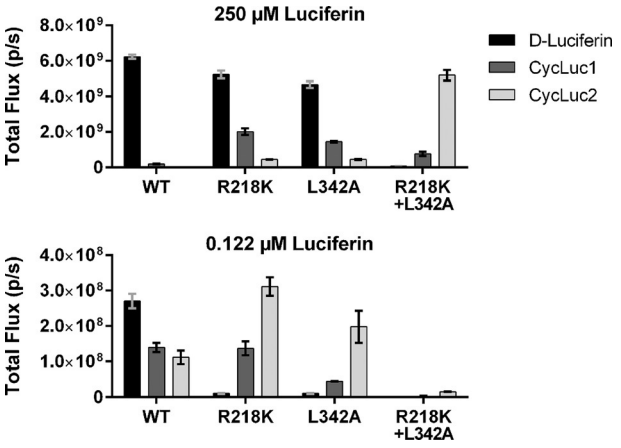

Figure 2. Photon flux from purified wild-type (WT) and mutant luciferases treated with high and low concentrations of luciferin substrate (see the Supporting Information for imaging details).

To evaluate the performance of these three luciferase mutants in vivo, each codon-optimized mutant was cloned into an adeno-associated viral (AAV) plasmid under the control of a CMV promoter,[9] and packaged into AAV9 vectors.[13,14] The AAV9-luciferase viruses were then used to transduce FVB mice in the brain striatum.[9] Unlike the use of luciferase-expressing tumor cells, this viral gene delivery method allows luciferase expression in endogenous mouse cells, over the lifetime of the mouse (well over a year). As we have previously described for AAV9 transduction with the WT luciferase, each luciferase-expressing mouse was imaged after intraperitoneal (i.p.) injection with a phosphate-buffered saline (PBS) solution of each luciferin.[4,9] These experiments were performed in the exact same set of mice, imaged with each substrate on sequential days.

For all of the mutant luciferases, brain bioluminescence was dramatically reduced when using D-luciferin compared to CycLuc1 and CycLuc2 (Figure 3). With the L342A luciferase, D-luciferin gave no signal, while both aminoluciferins gave a signal more than 50-fold above background. For the R218K luciferase, the D-luciferin signal was approximately 3-fold above background, but CycLuc1 and CycLuc2 were 40–50-fold brighter still. However, combining the R218K and L342A mutations did not yield synergism in vivo.

Although no photon flux above background was observed with D-luciferin for the double mutant, the photon flux was also substantially lower for CycLuc1 and CycLuc2 than for the individual point mutants (Figure S4). Thus, at least in the brain, the double mutant was less useful as a selective luciferase for these substrates compared to L342A or R218K alone.

Luciferin amides, sensors for the enzyme fatty acid amide hydrolase (FAAH), potentially offer a way to deliver luciferins into the brain more effectively.[4,15] Because of high FAAH activity in the brain, the amides are converted into their respective luciferins, and imaging can be performed with very low doses of the luciferin amide.[4] We therefore tested whether the amides of CycLuc1 and CycLuc2 (Figure 1) could be used to detect AAV transduction in the brain with the mutant luciferases (Figure 3). Remarkably, i.p. injection of just 0.4 μmol kg^{-1} CycLuc2-amide allowed imaging of L342A luciferase in the brain with a signal that was more than 400-fold above background, which is equivalent or better than its performance with WT luciferase (Figure S5). By contrast, 400 μmol kg^{-1} D-luciferin failed to yield any detectable signal (Figure 3). Luciferin comparison at low equimolar doses further revealed the relative potency of each analogue (Figure S6).

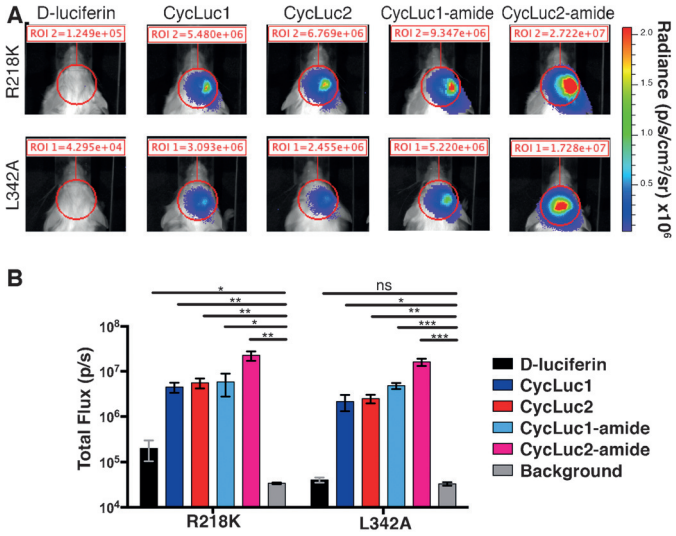

Figure 3. Mutant luciferase performance in the brains of live mice. A) Photon flux from a single AAV-transduced mouse compared after imaging with the indicated luciferases and luciferins (D-luciferin 400 μmol kg^{-1}; CycLuc1 20 μmol kg^{-1}; CycLuc2 10 μmol kg^{-1}; CycLuc1-amide,1 μmol kg^{-1}; CycLuc2-amide 0.4 μmol kg^{-1}). B) Photon flux from the region of interest (ROI), $n = 3$ mice for each mutant luciferase. * $p < 0.05$; ** $p < 0.01$; ns = not significant by t-test.

Firefly luciferase exhibits a great deal of structural plasticity, and accepts an almost bewildering array of molecules as substrates.[2,8] Although we demonstrate here that mutant luciferases can strongly discriminate against D-luciferin in vivo, the inherent promiscuity of firefly luciferase has posed a challenge for the construction of truly orthogonal luciferases. That is, we can identify mutated enzymes that will emit light with CycLuc2 but not D-luciferin, but as yet the converse has not been achieved. For most of the selective luciferases described thus far, we do not believe that the changes to the luciferin and luciferase mirror the classic "bump–hole" approach used for engineering ligand–receptor interactions.[16,17] Rather, the primary basis for substrate selectivity is likely a loss of affinity for the core features of D-luciferin. Because aminoluciferins bind with higher affinity, they remain viable substrates. Going forward, one approach to more selective luciferases would be to take advantage of homologous enzymes that are inherently more selective than firefly luciferase and may therefore offer guidance as to how more precise substrate selectivity can be achieved.[18] Herein, we also show that the interplay between the luciferin and ATP binding sites can play a role in discrimination between luciferins. Although leucine 342 is located far from the 6′-hydroxyl of D-luciferin (i.e., the site of the CycLuc1 and CycLuc2 modifications), mutation of this residue allows substrate selectivity in the brain. Future mechanistic work in vitro will be aimed at establishing the molecular basis for this interplay and uncovering how ATP binding affects the product inhibition seen with many luciferin analogues (Figure S2). Perhaps ATP can rebind luciferase prior to release of the oxyluciferin product, thereby resulting in a trapped complex.

With AAV-delivered reporters, we have a platform on which to evaluate luciferases, luciferins, and luciferin-based sensors in different animal models and tissues.[4,19–22] Despite the ability of the L342A luciferase to emit light when given sufficient quantities of D-luciferin and ATP in vitro, in the context of the live mouse brain, we do not detect a signal. On the other hand, strong brain bioluminescence is observed from the L342A luciferase when using the aminoluciferins, but not with the double mutant R218K/L342A, despite its higher peak performance in vitro. Based on these results, a prudent design consideration (and proxy) for in vivo activity is luciferase performance at low substrate concentration, rather than the maximal rate. In the development of new luciferins and luciferin-based sensors for in vivo use, it is also important to keep in mind the effect substrate modification has on the ability of the molecule to access particular tissues in the live animal.[15] Together, we expect that the judicious application of mutant luciferases and modified luciferins will greatly expand the repertoire of noninvasive optical imaging tools.

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