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A *Photinus pyralis* and *Luciola italica* Chimeric Firefly Luciferase Produces Enhanced Bioluminescence

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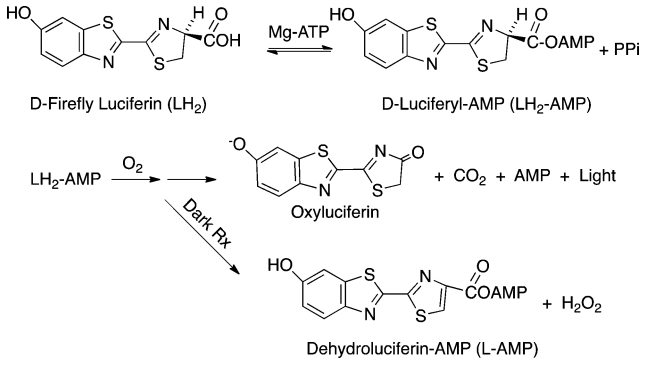
Supporting Information

ABSTRACT: We report the enhanced bioluminescence properties of a chimeric enzyme (PpyLit) that contains the N-domain of recombinant *Photinus pyralis* luciferase joined to the C-domain of recombinant *Luciola italica* luciferase. Compared to the *P. pyralis* enzyme, the novel PpyLit chimera exhibited 1.8-fold enhanced flash-height specific activity, 2.0-fold enhanced integration-based specific activity, 2.9-fold enhanced catalytic efficiency (k_{cat}/K_m), and a 1.4-fold greater bioluminescence quantum yield. The results of this study provide an underlying basis of this unusual example of a chimeric enzyme with enhanced catalytic properties that are not simply the sum of the contributions of the two luciferases.

It is well-established[1,2] that the beetle luciferases comprise one major group of a large superfamily of adenylating enzymes. The two-domain structure of the superfamily enzymes was originally recognized in the seminal crystallographic study of Brick[3] that described the *Photinus pyralis* luciferase large N-domain (residues 1−436) and small C-domain (residues 440−550) connected through the short hinge region of ^{437}ArgLeuLys^{439}. The luciferases (Luc) catalyze two half-reactions (Scheme 1): (1) the formation of LH_2-AMP and (2) a multistep oxidative process that produces light.[4]

There have been few reports of chimeric luciferases produced by connecting regions of different beetle luciferase sequences (see the Supporting Information), and among these, there are no examples of the enhancement of catalytic activity beyond that of wild-type Luc. As part of an ongoing study of Luc catalysis, we constructed PpyLit (Figures S1 and S2 of the Supporting Information), a chimeric firefly luciferase consisting of the N-domain (residues 1−439) of recombinant *P. pyralis* luciferase (PpyWT) joined to the C-domain (residues 442−548) of recombinant *Luciola italica* luciferase (LitWT).[5] The connecting hinge peptide, ^{437}ArgLeuLys^{439}, is identical in both enzymes. PpyWT and LitWT share 64% overall sequence identity, and in effect, the LitWT C-domain sequence introduced 27 changes, 23 amino acid substitutions and 4 deletions, into the full 550-amino acid PpyWT sequence (Figures S3 and S4 of the Supporting Information). We report here the first example of a Luc chimera with bioluminescence properties that exceed those of PpyWT.

Scheme 1. Reactions of Firefly Bioluminescence



CHARACTERIZATION OF PPYLIT

The chimeric PpyLit enzyme, which produces yellow-green light emission, has unusually enhanced bioluminescence properties (Table 1 and Table S1 of the Supporting Information). Compared to PpyWT, the novel PpyLit chimera exhibited (1) 1.8-fold enhanced flash-height (initial burst kinetics) specific activity, (2) 2.0-fold enhanced integration-based (total relative photon emission per 15 min) specific activity, and (3) 2.9-fold enhanced catalytic efficiency (k_{cat}/K_m) with respect to Mg-ATP. These results were quite unexpected as PpyWT is one of the most efficient bioluminescence systems known.

Table 1. Relative Specific Activities, Bioluminescence Quantum Yields, and Reaction Product Distributions

		relative Φ_{BL}[b]		products[c]	
Luc	specific activity[a]	LH_2	LH_2-AMP	oxy-LH_2	L-AMP
PpyWT	100 ± 4	100 ± 9	100 ± 9	77 ± 6	23 ± 1
LitWT	84 ± 2	96 ± 7	88 ± 3	72 ± 9	28 ± 2
PpyLit	180 ± 10	138 ± 6	138 ± 13	81 ± 4	21 ± 1
LitPpy	17 ± 1	66 ± 2	70 ± 4	70 ± 3	30 ± 3

[a]Flash-height-based specific activity with LH_2 and Mg-ATP. [b]Determined from integrated activity assays containing an excess of enzyme and Mg-ATP with limiting LH_2 or LH_2-AMP. Values are expressed relative to PpyWT, defined as 100. [c]Product distribution from the oxidative half-reaction. For additional details, see the Supporting Information.

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The traditional use of flash- or peak-height-based activity as a measure of initial reaction velocity expresses the maximal achievable rate of photon production resulting from the overall combined adenylation and oxidation steps (Scheme 1). The process is complex because the product oxyluciferin is a modest inhibitor (K_i = 0.5 μM),[6] and more importantly, the potent luciferase inhibitor L-AMP (K_i = 3.8 nM)[6] is formed in a "dark" side reaction (Scheme 1). Additionally, dehydroluciferin (L) (K_i = 0.5 ± 0.06 μM) can form by hydrolysis of L-AMP. Integration-based activity values are estimates of total light emitted and depend on the time to reach the maximal intensity as well as the rate of decay of the signals. Typically, as is the case for the greater (than PpyWT) integration-based specific activity of LitWT, prolonged light emission (Table S1 of the Supporting Information) was due to a longer decay time (3.3 min vs 0.21 min). However, this was not the case for PpyLit in which the enhanced total light emission was mainly due to the increased initial peak intensity (Table S1 of the Supporting Information and Figure 1). This was reflected in the catalytic

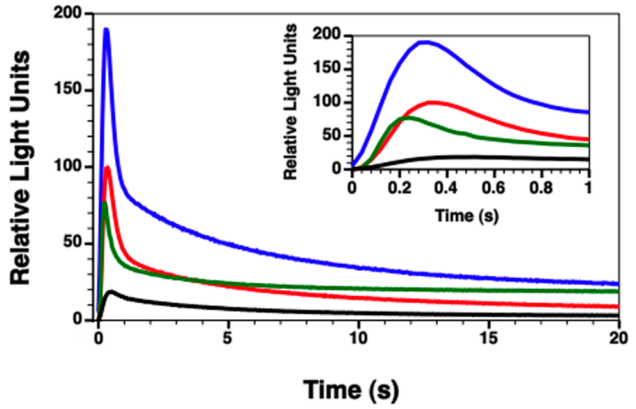

Figure 1. Bioluminescence time course of reactions of luciferases with LH_2 and Mg-ATP. Reactions (0.4 mL) in 25 mM glycylglycine buffer (pH 7.8) containing 0.8 μg of protein (PpyLit, blue; PpyWT, red; LitWT, green; and LitPpy, black) and 100−400 μM LH_2 were initiated by the injection of 0.12 mL of a solution of 9 mM Mg-ATP in the same buffer.

efficiency (k_{cat}/K_m) of PpyLit that was 2.9- and 7.7-fold higher than those of wild-type precursors PpyWT and LitWT, respectively, mainly because of its improved k_{cat} value (Table S1 of the Supporting Information).

To determine if the properties of this unusual chimeric protein were unique to the particular N- and C-domain combination of the wild-type luciferases, we made and evaluated LitPpy, containing LitWT residues 1−441 and PpyWT residues 440−550 (Table 1 and Table S1 and Figures S1 and S3 of the Supporting Information). The specific activity and catalytic efficiency of the LitPpy chimeric protein were significantly lower than those of PpyLit and the wild-type enzymes, reaffirming the importance of the particular domain arrangement of PpyLit. Additionally, we noted that the pH optimum of PpyLit was 8.0, quite similar to those of the wild-type enzymes. Furthermore, the bioluminescence emission spectrum (Figure S5 of the Supporting Information) of PpyLit (λ_{max} = 560 nm) at pH 7.8 was identical to that of PpyWT, but not that of LitWT (λ_{max} = 572 nm). Additionally, only PpyLit was resistant to the red shifting of light emission at low pH (6.0−7.0) that is characteristic of the true firefly luciferases, but not the railroad worm and click beetle enzymes (Table S1 and Figure S5 of the Supporting Information).[7]

■ RELATIVE BIOLUMINESCENCE QUANTUM YIELDS

To further characterize the properties of PpyLit and improve our understanding of the basis of the activity enhancement, we measured relative bioluminescence quantum yields (Φ_{BL}). Absolute quantum yields are technically very difficult to obtain, and recently, the value for PpyWT was remeasured[8] and determined to be 0.41 ± 0.074. The values listed in Table 1 were acquired with limiting LH_2 and excess Mg-ATP or with limiting LH_2-AMP and are reported relative to the corresponding values obtained with PpyWT. With excess enzyme present to ensure complete reaction of the substrates, the Φ_{BL} measures the intrinsic ability of a luciferase to convert a molecule of substrate into a photon. PpyLit had an ∼1.4-fold greater Φ_{BL} than PpyWT, suggesting that it has an absolute Φ_{BL} equal to ∼0.55, an indication that the chimeric protein may be considered a fundamentally altered version of PpyWT and LitWT. The relative Φ_{BL} values of PpyWT and LitWT were similar as were the flash-height specific activity and k_{cat} values (Table 1 and Table S1 of the Supporting Information).

As described by White and colleagues,[4] the relationship $\Phi_{BL} = \Phi_{RX}\Phi_{ES}\Phi_{FL}$ describes the dependence of Φ_{BL} on the reaction yield of emitter oxyluciferin (Φ_{RX}), the yield of oxyluciferin in the first electronically excited state (Φ_{ES}), and the fluorescence quantum yield of oxyluciferin (Φ_{FL}). On the basis of the results of a control experiment (Supporting Information), the increase in the Φ_{Fl} of enzyme-bound oxyluciferin did not account for the enhanced Φ_{BL} of PpyLit. Because the Φ_{ES} factor is not directly measurable, we focused on the Φ_{RX} value and the more straightforward issue of whether PpyLit may produce oxyluciferin in greater yield than PpyWT (Scheme 1).

■ REACTION PRODUCT ANALYSES

To simplify the LC/ESMS-based bioluminescence reaction product analysis, we used synthetic LH_2-AMP as the sole substrate (Scheme 1). The adenylate was incubated with an excess of each luciferase and the reaction products oxyluciferin and L-AMP were measured (Table 1 and the Supporting Information). Within experimental error, the mole percentage of LH_2-AMP converted into oxyluciferin (Φ_{RX}) was essentially the same (∼80%) for PpyLit and PpyWT. Apparently, the enhanced Φ_{BL} of PpyLit is predominantly attributable to a greater Φ_{ES}. Under substrate limiting conditions, the percentage of oxyluciferin produced in the excited state and/or the efficiency of photon emission was superior in PpyLit. Because PpyLit and PpyWT produced essentially the same amount of L-AMP inhibitor and because the two enzymes had similar rise and decay times with saturating levels of substrates, it is highly unlikely that the enhanced properties of PpyLit are caused by a reduced level of product inhibition. The similar inhibition characteristics of L-AMP and oxyluciferin for PpyWT (K_i = 3.8 ± 0.7 nM[6] and K_i = 0.50 ± 0.03 μM,[6] respectively) and PpyLit (K_i = 1.9 ± 0.6 nM and K_i = 0.75 ± 0.1 μM, respectively) support this notion.

■ CONCLUSIONS

The chimeric luciferase PpyLit has a bioluminescence quantum yield markedly (1.4-fold) greater than that of either of the wild-type enzymes from which it was constructed. To the best of our knowledge, this is the first report in which the bioluminescence

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quantum yield of a beetle luciferase has been enhanced. The basis for this improved fundamental property appears mainly to be the result of the enhanced efficiency of formation and/or radiative decay of the emitter oxyluciferin. On the basis of comparisons with PpyWT, the functional changes in PpyLit do not appear to be related to differences in secondary structure or overall stability assessed by circular dichroism (Table S2 of the Supporting Information) and denaturation studies (Figure S6 of the Supporting Information). We hypothesize that the augmented Φ_{ES} results from a conformation of the enzyme in which the N- and C-domains interact to provide a more favorable environment for electronically excited state oxyluciferin. Additional studies are in progress to identify the key residues associated with the enhanced PpyLit properties and to produce novel luciferase variants for reporter, imaging, and other applications.

■ ASSOCIATED CONTENT

Supporting Information

Detailed materials and methods, additional introduction and discussion, and tables and figures illustrating the sequences and properties of the wild-type and chimeric luciferases. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

L, dehydroluciferin; L-AMP, dehydroluciferyl-AMP; LH_2, D-firefly luciferin; LitWT, recombinant *L. italica* luciferase with changes Lys547Gly and Met548Gly; LH_2-AMP, luciferyl-AMP; LitPpy, chimeric protein comprised of LitWT residues 1−441 and PpyWT residues 440−550; Luc, firefly luciferase; $OxyLH_2$, oxyluciferin; PpyWT, recombinant *P. pyralis* luciferase; PpyLit, chimeric protein comprised of PpyWT residues 1−439 and LitWT residues 442−548.

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