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Construction of a new firefly bioluminescence system using L-luciferin as substrate

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Abstract—L-Luciferin can be converted into D-luciferin with an enzyme/co-factor system consisting of firefly luciferase, an esterase, ATP, Mg^{2+}, and coenzyme A. By this means, a new firefly bioluminescence system can be constructed that uses L-luciferin as the substrate.

Bioluminescence systems are widely used in various biological, medical, and health science fields because highly sensitive devices are available to detect the light emitted. Of these, the firefly bioluminescence system that requires ATP as a co-factor is particularly useful for the detection of various biological events involving ATP and the detection of bacterial contamination of food, water, air, and various environments.[1,2] The firefly bioluminescence system is also useful for monitoring gene expression in promoter assays and for protein localization, with measurement of the emitted light.[2–7] In the bioluminescence reaction, firefly D-luciferin is first converted to luciferyl adenylate by firefly luciferase (EC 1.13.12.7) in the presence of ATP and Mg^{2+}. The luciferyl adenylate is then oxidized by luciferase with molecular oxygen, yielding a yellow–green light (λ_{max} = 550–570 nm) with the formation of oxyluciferin, CO_2, and AMP.[8–11] D-Luciferin, the specific substrate for the bioluminescence reaction, has the same chirality, with a stereogenic centre, as unnatural D-cysteine. The enantiomer L-luciferin has the same chirality as natural L-cysteine, and behaves as a competitive luciferase inhibitor, as do dehydroluciferin, fatty acids, and lipoic acid.[12–15] Whereas several research groups have reported that no light was produced from L-luciferin,[16–19] Lembert noted that L-luciferin produced weak light very slowly under the usual bioluminescence conditions (firefly luciferase, L-luciferin, ATP, and Mg^{2+}).[20] More recently, light production from luciferyl-CoA and AMP in the presence of firefly luciferase has been reported.[21] Firefly luciferase is known to have coenzyme A (CoA-SH) ligase activity for dehydroluciferin and fatty acids.[13,22,23] We have recently demonstrated that firefly luciferase exhibits a bimodal action depending on luciferin chirality under normal aerobic conditions: D-luciferin-specific bioluminescence activity and L-luciferin-specific acyl-CoA synthetase activity.[13] The inhibition of D-luciferin bioluminescence by L-luciferin, dehydroluciferin, or fatty acids is known to be abolished by the addition of CoA-SH to the reaction mixture.[10–12]

Based on previous reports[1–23] including our recent finding,[13] we anticipated that the epimerization followed by hydrolysis of luciferyl-CoA derived from L-luciferin by the action of firefly luciferase may yield bioluminescent-active D-luciferin, which would allow us to produce continuous light using L-luciferin (Scheme 1). We found that luciferin methyl ester was easily racemized in 50 mM Hepes buffer at pH 7.6.[13] As mentioned above, firefly luciferase exhibits L-luciferin-specific, CoA synthetase activity.[13] Therefore, a thioester-directed hydrolytic enzyme could hydrolyze epimerized luciferyl-CoA

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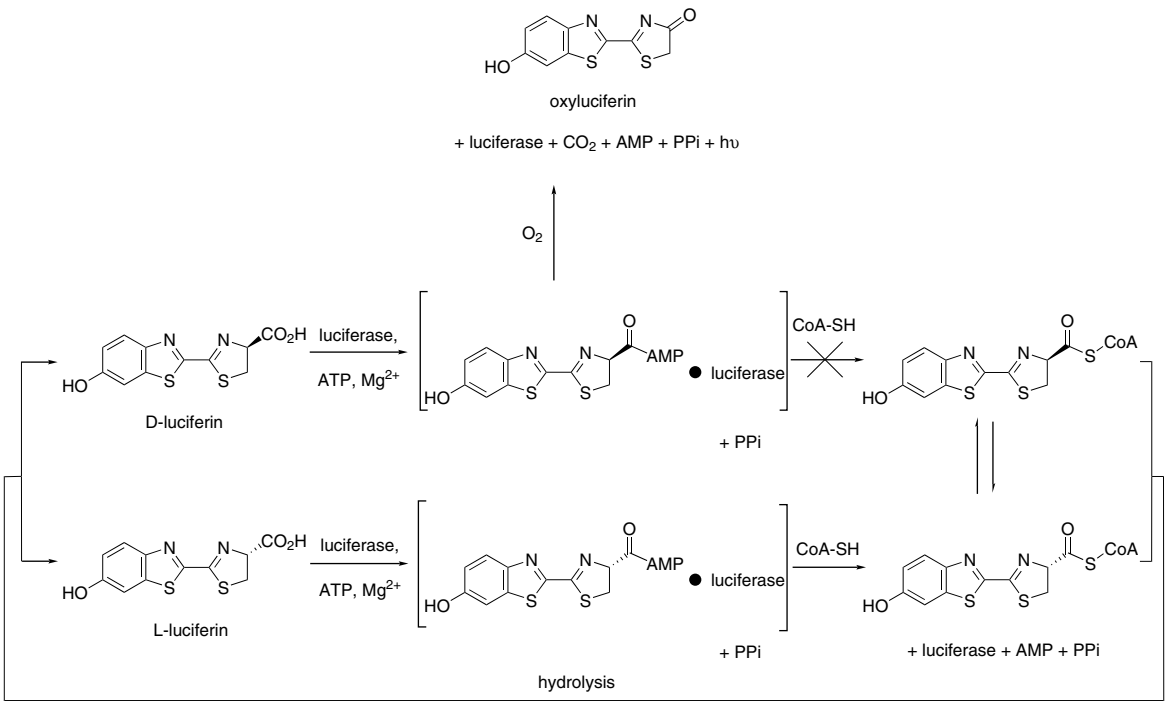

Scheme 1. Proposed mechanism for the production of D-luciferin from L-luciferin via luciferyl-CoA.

to yield D- and L-luciferin. Whereas the regenerated L-luciferin would be rapidly reconverted to luciferyl-CoA, D-luciferin could be used for the light-producing reaction. We report here the development of a new firefly bioluminescence system using L-luciferin, the antipode of D-luciferin.

An esterase from porcine liver (from Sigma–Aldrich Chemical Co., Milwaukee, WI) was used to hydrolyze the luciferyl-CoA thioester, because the enzyme is flexible in its recognition of substrates. The esterase hydrolyzed luciferyl-CoA (data not shown). The reaction mixture (400 μL) contained L-luciferin (50 μM), CoA-SH (500 μM), ATP (1.5 mM), $MgSO_4$ (4 mM), luciferase (10 μg/mL), and esterase (0.2 mg/mL) in 0.1 M Tris buffer at pH 8.0. The reaction was initiated by the addition of a solution of ATP and $MgSO_4$. The reaction was monitored by high-performance liquid chromatography (HPLC) analysis of D- and L-luciferin using a chiral column (Chiralcel OD-RH, 4.6 × 150 mm; Daicel Chemical Industries, Ltd, Osaka, Japan) eluted with 27% acetonitrile containing 0.1% TFA, by measuring fluorescence (excitation 330 nm, emission 530 nm) (Fig. 1).

As shown in Figure 1, the quantity of L-luciferin rapidly decreased (Fig. 1, A and B) and luciferyl-CoA appeared instantaneously, as reported previously.[13] After an induction period, the amount of D-luciferin gradually increased. This indicates that D-luciferin was generated from L-luciferin in the presence of firefly luciferase, esterase, CoA-SH, ATP, and Mg^{2+}. In the absence of CoA-SH in this reaction, L-luciferin was essentially not consumed and its concentration remained almost constant during the observation period, indicating that CoA-SH is the essential component in the production of D-luciferin (Fig. 1, C). On the other hand, luciferyl-CoA was not produced from D-luciferin by firefly luciferase.[13] Therefore, L-luciferin was not produced directly from D-luciferin by the action of firefly luciferase and esterase. This means that this conversion reaction is enantio-specific, and this enantio-specificity is due to the strict chiral recognition capacity of firefly luciferase. These results indicate that L-luciferin was first converted into L-luciferyl-CoA by the action of the luciferase serving as L-luciferin-selective CoA ligase, in the presence of ATP, Mg^{2+}, and CoA-SH (Scheme 1).[13] The epimerization of the derived L-luciferyl-CoA and the subsequent hydrolysis of the epimerized luciferyl-CoA with the esterase furnished D-luciferin along with L-luciferin.

Since D-luciferin was found to be always present in the reaction mixture at a certain concentration over several hours as shown in Figure 1, the continuous light production was observed through several hours (data not shown). Most of the luciferin used was present as luciferyl-CoA (luciferyl-CoA:L-luciferin:D-luciferin = 96.5:0.8:2.7, 10 min), because the reaction rate of the luciferyl-CoA hydrolysis with the porcine liver esterase was slower than CoA ligase reaction with the firefly luciferase. Therefore, the amount of light emission should be controlled with the hydrolysis activity of esterase co-present in the reaction mixture.

L-Luciferin is known to inhibit the bioluminescence activity of D-luciferin, and the binding ability of L-luciferin to firefly luciferase was found to be three times stronger than that of D-luciferin.[20] The inhibition of D-luciferin bioluminescence by L-luciferin is known to be abolished by the addition of CoA-SH to the reaction mixture.[10–13] Thus, L-luciferin regenerated from the

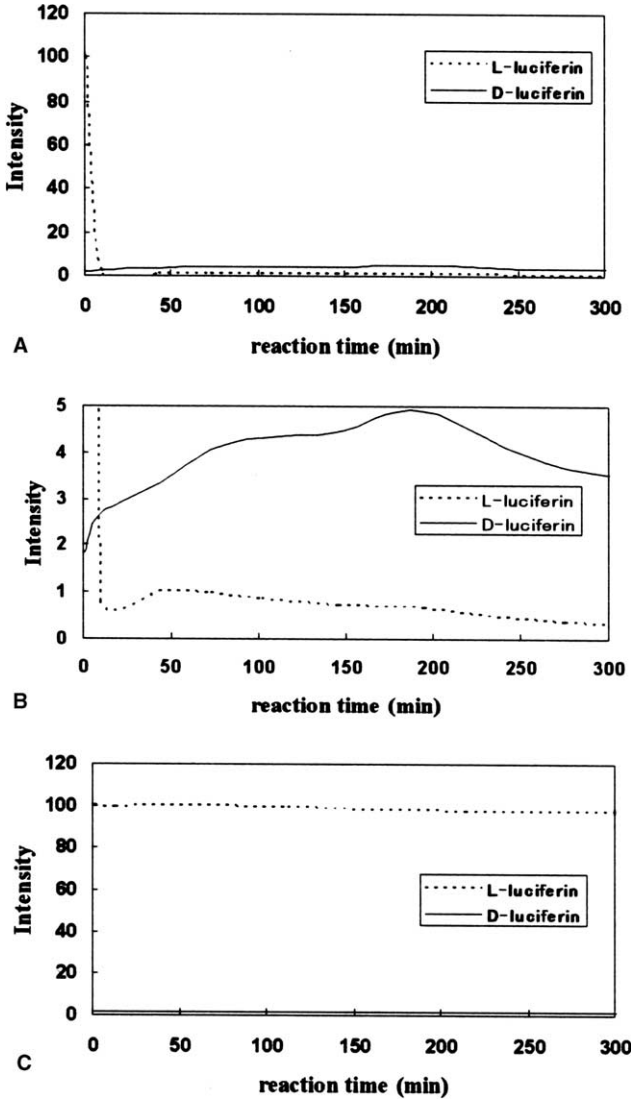

Figure 1. The time course of the change in fluorescence intensity of L- and D-luciferin in the reaction of L-luciferin with ATP, $MgSO_4$, luciferase, and esterase in the presence of CoA-SH (A and B) and in the absence of CoA-SH (C). B is a magnification of the figure in A. The reaction conditions were as follows: (A) L-luciferin (50 μM), CoA-SH (500 μM), ATP (1.5 mM), $MgSO_4$ (4 mM), luciferase (10 μg/mL), and esterase (0.2 mg/mL) in 0.1 M Tris buffer at pH 8.0. (B) The reaction conditions in the absence of CoA-SH. The reaction was monitored by HPLC analysis of D- and L-luciferin using a chiral column (Chiralcel OD-RH, 4.6 × 150 mm; Daicel Chemical Industries, Ltd, Osaka, Japan) eluted with 27% acetonitrile containing 0.1% TFA, by measuring fluorescence (excitation 330 nm, emission 530 nm). L-Luciferin and D-luciferin were eluted at 7.1 and 8.4 min, respectively.

hydrolysis of the epimerized luciferyl-CoA does not inhibit the bioluminescence activity of D-luciferin, because the regenerated L-luciferin was immediately reconverted into luciferyl-CoA with firefly luciferase and the resulting luciferyl-CoA is removed from the active site.

The bioluminescence activity of the present reaction system using L-luciferin at various luciferase concentrations exhibited linearity ($R^2 > 0.99$) with the luciferase concentration used (Fig. 2), indicating that the present method should be useful as a promoter assay in which

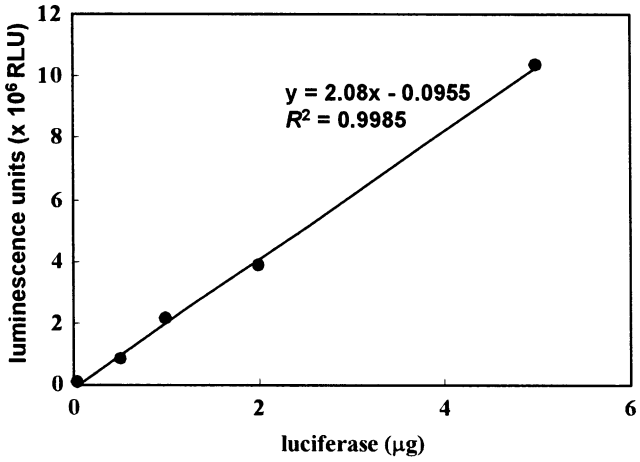

Figure 2. Assay of firefly luciferase using L-luciferin as the substrate. The reaction conditions were as follows: L-luciferin (50 μM), CoA-SH (500 μM), ATP (1.5 mM), $MgSO_4$ (4 mM), luciferase (see figure), and esterase (0.2 mg/mL) in 0.1 M Tris buffer at pH 8.0. The vertical axis indicates the integration of luminescence from 1 to 50 s.

luciferase concentration is monitored as a reporter enzyme.

In conclusion, we have identified a novel route of D-luciferin formation from L-luciferin *in vitro* that allows the construction of a new firefly bioluminescence system using L-luciferin, which is available cheaply from natural L-cysteine. In living cells, CoA-SH, ATP, Mg^{2+}, and some kind of esterase or thioesterase are present as ubiquitous cell components. Therefore, L-luciferin may be used to quantitatively monitor luciferase concentration as a reporter enzyme for gene expression in living cells.

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References and notes

1. Petty, R. D.; Sutherland, L. A.; Hunter, E. M.; Cree, I. A. *J. Biolumin. Chemilumin.* **1995**, *10*, 29–34.
2. Fukuda, J.; Fujita, Y.; Ohsawa, K. *J. Neurosci. Methods* **1983**, *8*, 295–302.
3. Keller, G. A.; Gould, S.; DeLuca, M.; Subramani, S. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 3264–3268.
4. Pazzagli, M.; Devine, J. H.; Peterson, D. O.; Baldwin, T. O. *Anal. Biochem.* **1992**, *204*, 315–323.
5. Badia, E.; Duchesne, M. J.; Pons, M.; Nicolas, J. C. *Anal. Biochem.* **1994**, *217*, 333–335.
6. Aflalo, C. *Int. Rev. Cytol.* **1991**, *130*, 269–323.
7. Wood, K. V. *J. Biolumin. Chemilumin.* **1990**, *5*, 107–114.
8. McElroy, W. D.; Seliger, H. H.; White, E. H. *Photochem. Photobiol.* **1969**, *10*, 153–170.
9. White, E. H.; Rapaport, E.; Seliger, H. H.; Hopkins, T. A. *Bioorg. Chem.* **1971**, *1*, 92–122.

10. DeLuca, M. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1976**, *44*, 37–68.
11. Wood, K. V. *Photochem. Photobiol.* **1995**, *62*, 662–673.
12. Sillero, A.; Günther Sillero, M. A. *Pharmacol. Ther.* **2000**, *87*, 91–102.
13. Nakamura, M.; Maki, S.; Amano, Y.; Ohkita, Y.; Niwa, K.; Hirano, T.; Ohmiya, Y.; Niwa, H. *Biochem. Biophys. Res. Commun.* **2005**, *331*, 471–475.
14. Ueda, I.; Suzuki, A. *Biophys. J.* **1998**, *75*, 1052–1057.
15. Niwa, K.; Ohmiya, Y. *Biochem. Biophys. Res. Commun.* **2004**, *323*, 625–629.
16. White, E. H.; McCapra, F.; Field, G. F.; McElroy, W. D. *J. Am. Chem. Soc.* **1961**, *83*, 2402–2403.
17. McElroy, W. D.; Seliger, H. H. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **1962**, *21*, 1006–1012.
18. White, E. H.; McCapra, F.; Field, G. F. *J. Am. Chem. Soc.* **1963**, *85*, 337–343.
19. Branchini, B. R.; Hayward, M. M.; Bamford, S.; Brennan, P. M.; Lajiness, E. J. *Photochem. Photobiol.* **1989**, *49*, 689–695.
20. Lembert, N. *Biochem. J.* **1996**, *317*, 273–277.
21. Fraga, H.; Fontes, R.; Esteves da Silva, J. C. G. *Angew. Chem., Int. Ed.* **2005**, *44*, 3427–3429.
22. Airth, R. L.; Rhodes, W. C.; McElroy, W. D. *Biochem. Biophys. Acta* **1958**, *27*, 519–532.
23. Oba, Y.; Ojika, M.; Inouye, S. *FEBS Lett.* **2003**, *540*, 251–254.