Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytical Biochemistry 345 (2005) 140–148
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Red- and green-emitting firefly luciferase mutants for bioluminescent reporter applications

Bruce R. Branchini [a,*], Tara L. Southworth [a], Neelum F. Khattak [a], Elisa Michelini [b], Aldo Roda [b]

[a] *Department of Chemistry, Connecticut College, New London, CT 06320, USA*
[b] *Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, Bologna 40126, Italy*

Received 4 May 2005
Available online 8 August 2005

Abstract

Light emission from the North American firefly *Photinus pyralis*, which emits yellow–green (557-nm) light, is widely believed to be the most efficient bioluminescence system known, making this luciferase an excellent tool for monitoring gene expression. Here, we present studies leading to the production of a set of red- and green-emitting luciferase mutants with bioluminescent properties suitable for expanding the use of the *P. pyralis* system to dual-color reporter assays, biosensor measurements with internal controls, and imaging techniques. Using a combination of mutagenesis methods, we determined that the Ser284Thr mutation was sufficient to create an excellent red-emitting luciferase with a bioluminescence maximum of 615 nm, a narrow emission bandwidth, and favorable kinetic properties. Also, we developed a luciferase, containing the changes Val241Ile, Gly246Ala, and Phe250Ser, whose emission maximum was blue-shifted to 549 nm, providing a set of enzymes whose bioluminescence maxima were separated by 66 nm. Model studies demonstrated that in assays using a set of optical filters, the luciferases could be detected at the attomole level and seven orders of magnitude higher. In addition, in the presence of the Ser284Thr enzyme serving as a control, green light emission could be measured over a 10,000-fold range. The results presented here with the *P. pyralis* mutants provide evidence that simultaneous multiple analyte assay development is feasible with these novel proteins that require only a single substrate.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Bioluminescence; Firefly; Luciferase; Mutagenesis; Imaging; Reporter; Dual color; Red emission; Multiplex assay

Luciferase from the North American firefly *Photinus pyralis* (Luc)[1] is a well-characterized enzyme that catalyzes the emission of yellow–green light [1,2]. Luc first converts the substrates firefly luciferin (LH_2), a heterocyclic carboxylic acid, and Mg-ATP into the corresponding luciferyl-adenylate (Eq. (1)). This reactive intermediate combines with molecular oxygen at the luciferase-active site to produce an electronically excited state product (Eq. (2)), which rapidly emits a photon of visible light (Eq. (3)):

$$\mathrm{Luc} + \mathrm{LH_2} + \mathrm{ATP} \overset{\mathrm{Mg^{2+}}}{\leftrightarrows} \mathrm{Luc \cdot LH_2\text{-}AMP} + \mathrm{Ppi}, \quad (1)$$

$$\mathrm{Luc \cdot LH_2\text{-}AMP} + \mathrm{O_2} \rightarrow \mathrm{Luc \cdot Oxyluciferin^*} + \mathrm{AMP} + \mathrm{CO_2}, \quad (2)$$

$$\mathrm{Luc \cdot Oxyluciferin^*} \rightarrow \mathrm{Luc \cdot Oxyluciferin} + h\upsilon. \quad (3)$$

* Corresponding author. Fax: +1 860 439 2479.
E-mail address: brbra@conncoll.edu (B.R. Branchini).

[1] *Abbreviations used:* Luc, *Photinus pyralis* luciferase (EC 1.13.12.7); LH_2, D-firefly luciferin; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; WT, recombinant *P. pyralis* luciferase containing the additional N-terminal peptide GlyProLeuGlySer–; LB, Luria–Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; CBA, 50 mM Tris–HCl (pH 7.0) containing 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol, 0.8 M ammonium sulfate, and 2% glycerol; BSA, bovine serum albumin.

0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2005.07.015

The generation of light from LH_2 is highly efficient [3], affording great sensitivity for the detection of the luciferase protein using available light-measuring technology. Attomole amounts of the enzyme can be detected above background cell contamination, which is essentially absent [4], making luciferase an excellent candidate for additional novel applications requiring reporter gene assays [5] and in vivo bioluminescence imaging [6]. Luciferases already have been used to study gene delivery [7], gene silencing [8], and real-time imaging of luciferase expression in live animals [9]. In addition, several whole cell-based biosensors and bioassays have been developed [10–13] for the detection of various analytes using native and recombinant biosensing cells with reporter luciferase proteins (firefly and bacterial) and green fluorescent protein (GFP). The main drawback encountered in the analytical application of these biosensors is the high variability of the response caused by sample matrix or environmental conditions. The analyte-dependent reporter protein synthesis can be influenced by a variety of changes in the cell's environment, resulting in nonspecific effects. To overcome this problem, an internal or external reference signal can be introduced to correct the analytical response and consequently isolate the analytical signal from nonspecific interferences. This can be achieved by introducing a second reporter gene to correct the signal accordingly to cell vitality and sample matrix effects.

Assays using luciferase as a reporter are sensitive, quantitative, rapid, reproducible, and relatively easy to perform [14,15]. However, because of the complexity of cellular genetic regulation, interferences can make it difficult to characterize a specific physiological response. A second "control" reporter can be introduced to improve the specificity and precision of genetic regulation measurements. The Promega Dual-Luciferase Reporter Assay System, which requires sequential measurement of both firefly and *Renilla* luciferases in one sample, is an example of a dual-reporter assay system based on bioluminescence. Drawbacks of this system include the necessity to add a reagent to stop one reaction before adding a second substrate and the expression of two dissimilar enzymes. Recently, reporter assay systems using red- and green-emitting luciferases from the Jamaican click beetles [16] and *Phrixothrix* railroad worms [17–19] have been reported. Although these methods are promising, the sensitivity of these dual-color reporters might not be sufficient for some applications.

Our objective was to produce mutants of luciferase with bioluminescent properties suitable for improved dual-color reporter assays, biosensor measurements, and imaging techniques based on the *P. pyralis* enzyme widely believed to be the most efficient bioluminescence system known [3]. Few red-emitting Luc mutants for bioluminescence imaging have been reported [20], and these have not been well characterized. A previous random mutagenesis study in our laboratory [21] identified an efficient red-emitting luciferase containing three mutations: Val241Ile, Ser284Thr, and Ile351Ala. Full characterization of the individual mutants has enabled us to develop a new set of red- and green-emitting luciferases with bioluminescence emission spectra distinguishable by optical filters. Here, we present the findings of studies on the development of luciferases with suitable bioluminescent properties for demanding imaging and reporter gene applications, including the monitoring of two gene expressions in the same cell system.

Materials and methods

Materials

The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) (Sigma); LH_2 (Biosynth); restriction endonucleases (New England BioLabs); mutagenic oligonucleotides (Invitrogen); glutathione-*S*-transferase (GST) purification module, pGEX-6P-2 expression vector (Amersham Biosciences); GeneMorph PCR Mutagenesis and QuikChange Site-Directed Mutagenesis kits (Stratagene). WT (recombinant *P. pyralis* luciferase containing the additional N-terminal peptide GlyProLeuGlySer–) and Val241Ile/Ser284Thr/Ile351Ala in the pGEX-6P-2 plasmid were prepared as reported previously [21,22].

General methods

A detailed description of the equipment used to determine bioluminescence activity by light assays was described previously [23]. All luciferases in pGEX-6P-2 plasmids were expressed in *Escherichia coli* strain BL21 at 22 °C and were purified by the method reported previously [21]. The Ile351Ala mutational library was created and screened as described previously [21].

Mass spectral analyses of the proteins were performed by tandem HPLC–electrospray ionization mass spectrometry using either a PerkinElmer series 200 HPLC system and a Sciex ABI150A mass spectrometer or a ThermoFinnigan Surveyor HPLC system and a ThermoFinnigan LCQ Advantage mass spectrometer. The calculated molecular masses (in daltons) were as follows: WT, 61,157; V241I, 61,171; S284H, 61,207; S284I, 61,183; S284N, 61,184; S284T, 61,171; V241I/S284T, 61,185; V241I/S284T/I351A, 61,143; G246A/F250S, 61,111; and V241I/G246A/F250S, 61,125. All of the determined mass values were within the allowable experimental error of the calculated values. The mutations of all luciferase genes were verified by DNA sequencing performed at the W. M. Keck Biotechnology Laboratory at Yale University.

Download English Version:

https://daneshyari.com/en/article/10536284

Download Persian Version:

https://daneshyari.com/article/10536284

Daneshyari.com