Biochimica et Biophysica Acta 1864 (2016) 1818–1826

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Color-shifting mutations in the C-domain of *L. mingrelica* firefly luciferase provide new information about the domain alternation mechanism

Yulia Modestova *, Natalia N. Ugarova

Department of Chemical Enzymology, Faculty of Chemistry, Moscow State University, Moscow 119991, Russia
Lumtek LLC, ul. Vorob'ievy Gory 1/77, Moscow 119992, Russia

ARTICLE INFO

Article history:
Received 1 June 2016
Received in revised form 9 September 2016
Accepted 14 September 2016
Available online 16 September 2016

Keywords:
Firefly luciferase
Bioluminescence
pH-sensitivity
C-domain
Domain alternation mechanism

ABSTRACT

We identified three color-shifting mutations—Phe467Ser, Glu490Val, and Glu490Lys—in the C-domain of the wild-type recombinant *L. mingrelica* luciferase. These mutations had moderate effect on the specific activity and thermal stability of the enzyme but changed the pH-dependence of its bioluminescence spectra. We constructed the model structures of the enzyme in three known conformations (open, adenylation, and oxidation conformation). The structural analysis and experimental data provided no evidences that these residues participate in structure-forming interactions in the open or oxidation conformation or that their mutations alter the overall structure of the enzyme. Given that the bioluminescence spectra reflect the microenvironment of the emitter (oxyluciferin in an electronically excited state), we concluded that the mutated residues affect the active site during the emission of light via short-range interactions. We found that it is only in the adenylation conformation that the residues Phe467 and Glu490 approach the N-domain, whereas the domain rotation associated with the oxidation conformation completely removes them from the active site. Therefore, the emission most likely occurs from the adenylation conformation.

1. Introduction

Firefly luciferase catalyzes the bioluminescent oxidation of firefly luciferin, in which ATP is the second substrate [1,2]. This reaction is characterized by the highest quantum yield among the known bioluminescent systems [3,4]. Firefly luciferase has a variety of applications: it acts as a reporter for studying different biological processes in vivo [5,6], as a label in various bioanalytical methods [7–9], as an ultrasensitive detector of ATP in bioluminescence ATP assays [10], or as a detector of coenzyme A [11] or free fatty acids [12]. It can also be used as an excitation source in the photodynamic therapy of cancer [13].

Firefly luciferase is a class I adenylation enzyme [14]; it shares three common characteristic features of this class [15]. First, it is a two-domain enzyme: a small C-domain is connected to a large N-domain, comprising most of the catalytic residues, via a flexible linker. Second, its catalytic reaction proceeds in two steps: (1) adenylation of the main substrate (firefly luciferin) and (2) subsequent transformation of the produced adenylate (oxidation of luciferyl adenylate). Third, the enzyme uses a domain alternation mechanism: the relative positions of its domains change during the reaction to provide optimum microenvironments for each of the reaction steps. Different research groups demonstrated that the C-domain rotation is crucial for coupling the adenylation and oxidation steps of the reaction [16–18].

According to the studies performed using crystallography [19–21] and mutagenesis [17,18,22], the enzyme adopts three distinct conformations: an open conformation of the free enzyme and two closed catalytic conformations (adenylation and oxidation conformations) corresponding to each of the reaction steps. To switch from one catalytic conformation to the other, the C-domain rotates around the N-domain, which comprises most of the active site, by the angle of ~140° [15]. This rotation successively brings each of the C-domain catalytic residues—Lys529 [23] and Lys443 [22] in *P. pyralis* luciferase—into the active site at the adenylation and oxidation steps of the reaction, respectively. Mutations of these residues can produce luciferases that catalyze only one of the reaction steps [16]. The firefly luciferase cross-linked in the oxidation conformation is unable to catalyze the adenylation of firefly luciferin; however, it can efficiently catalyze the bioluminescent oxidation of synthetic luciferyl adenylate [18].

The spectrum of light generated by the bioluminescence system of firefly luciferases varies widely (λ_{max} = 536–623 nm) [24]. It reflects the states of the emitter and its microenvironment during the emission of light; these states, in turn, depend on such parameters as pH, temperature, and the overall structure of the enzyme [25–28]. This feature makes firefly luciferase an interesting model to study structure–

* Corresponding author at: Department of Chemical Enzymology, Faculty of Chemistry, Moscow State University, Moscow 119991, Russia.
E-mail address: jmodestova@yahoo.com (Y. Modestova).

http://dx.doi.org/10.1016/j.bbapap.2016.09.007
1570-9639/

function relationships in proteins, since the slightest impact on the active site or its neighboring residues can change the bioluminescence spectrum.

Over the years, researchers described many color-shifting mutations in the N-domain providing interesting material for the ongoing studies of the color modulation in firefly luciferases [27,29–31]. Surprisingly, the role of the C-domain in this mechanism is mostly overlooked. Though several studies describe C-domain mutations, some of them had been conducted before the crystal structures of firefly luciferase in different conformations were obtained, so the authors had not been able to link the enzyme structure to the gathered data [32,33]. Other papers describe mutations that had strong effects on the structure and the catalytic parameters of the enzyme [34–37]. For instant, the color-shifting mutations of the Glu457 residue reduced the activity of the wild-type *L. mingrelica* luciferase to such extent that an enzyme with the stabilized N-domain had to be used to study these mutations in detail [36,37].

In the present study, we used the wild-type recombinant *L. mingrelica* luciferase to find color-shifting C-domain mutations that specifically affect the bioluminescent properties of the system without impairing the catalytic process or the enzyme structure. The study of color-shifting C-domain mutations could shed light on the role of the C-domain in color modulation and reveal new aspects of the domain alternation mechanism.

2. Materials and methods

2.1. Materials

Na-ATP (cat. no. A2383), bovine serum albumin, dithiothreitol (DTT), and yeast extract (cat. no. Y-0500) were from Sigma-Aldrich (USA). D-luciferin was from Lumtek (Russia). TaqSE DNA polymerase was from SibEnzyme (Russia). T4 DNA ligase and the restriction enzymes *Apa*I, *Bgl*II, and *Nhe*I were from Thermo Scientific (USA). Bacto-tryptone was from Becton Dickinson and lactose 1-hydrate was from Panreac (Spain). Competent *E. coli* cells (strains Xl1-Blue and BL21 (DE3) CodonPlus) were prepared and transformed according to the method developed by Tu et al. [38]. Other chemicals were of analytical grade. Solutions were prepared using a Milli-Q water (Millipore, France).

2.2. Random mutagenesis of the C-domain region

We used the plasmid pLR4 (GenBank no. HQ007052) encoding the gene of the recombinant *L. mingrelica* luciferase [31]. In this plasmid, the region between the *Bam*HI and *Apa*I restriction sites corresponds to the luciferase fragment comprising the entire C-domain and 48 amino acids associated with the interconnecting flexible loop and a small fraction of the N-domain. This region was subjected to random mutagenesis as described previously [36]. The PCR reaction mixture (50 μL) comprised 10 mM Tris-HCl (pH 8.3 at 25 °C), 50 mM KCl, 7 mM $MgCl_2$, 0.3 mM $MnCl_2$, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 20 pmol of primers f_XhoI and r_T7term [36], ~2 fmol of pLR4, and 2.5 units of Taq DNA-polymerase. PCR was performed in a Mastercycler Gradient (Epppendorf) under the following conditions: 95 °C, 1 min; 1 min at 94 °C, 1.3 min at 53 °C, and 1 min at 72 °C (30 cycles); then, finally, 10 min at 72 °C. The expected error frequency under these conditions is approximately one amino acid per 500 base pairs [39]. The PCR product was gel purified using a Cleanup Mini kit (Evrogen, Russia) and digested with the *Bgl*II and *Apa*I restriction enzymes. The resulting product was gel purified and ligated into the vector that was obtained by treating the pLR4 plasmid with the same restriction enzymes. A typical ligation mixture (20 μL) containing ~20 ng insert, ~50 ng vector, 1× T4 DNA ligase buffer, 1 Weiss Unit T4 DNA ligase, and 5% (w/v) PEG-8000 was incubated at room temperature for 1 h. *E. coli* XL1-Blue competent cells were transformed with the ligation mixture and plated onto LB agar plates containing 100 μg/mL ampicillin. Each transformation produced approximately 1500 colonies per plate.

2.3. Screening of mutant proteins

The *E. coli* colonies expressing mutant luciferase genes were grown overnight at 37 °C on LB agar plates containing 100 μg/mL ampicillin and then incubated for 6–10 h at room temperature. The intensity of bioluminescence was registered in vivo by photographing the colonies upon treating them with 0.5 mM luciferin solution in 0.1 M Na-citrate buffer (pH 5.5). The photographs were taken in a dark room with a PowerShot A530 digital camera (Canon). The colonies were screened for intense green or red bioluminescence. The most promising colonies were transferred onto a new LB agar plate, grown overnight at 37 °C, incubated at room temperature, and screened again. The plasmids from those colonies that demonstrated the most pronounced color shift and the highest activity of bioluminescence were isolated using a Plasmid Miniprep kit (Evrogen, Russia) and sequenced. The sequencing was performed using an ABI PRISM® BigDye™Terminator v.3.1 kit with the subsequent analysis of the products on an ABI PRISM3730 Avant automatic DNA sequencer.

2.4. Luciferase expression and purification

Mutant luciferase genes were cloned in the pETL7 plasmid (GenBank no. HQ007050) [31]; the presence of the mutations was confirmed by sequencing as described above. The pETL7 plasmid encodes the luciferase, in which a C-terminal AKM peptide is changed to SGPVEHHHHHH and a MASK sequence is added to the N-terminus. Enzymes were expressed in *E. coli* BL21 (DE3) CodonPlus cells according to the lactose autoinduction protocol [40] and purified on a 1-mL Ni-IDA column (GE Healthcare, Sweden). The purified enzymes were stored at 4 °C in 20 mM sodium-phosphate buffer (pH 7.5) containing 500 mM NaCl, 300 mM imidazole, and 2 mM EDTA. Enzyme concentrations were determined by absorbance at 280 nm using the absorption coefficient $A = 0.56\ M^{-1}\ cm^{-1}$ for a 0.1% solution of *L. mingrelica* luciferase; the absorption coefficient was calculated based on the amino acid sequence of the protein [41].

2.5. Enzyme activity

The intensity of light emitted during the luciferase reaction was measured at room temperature using an FB12 luminometer (Zylux, USA) equipped with a Diluter 2075 injector (LKB, Sweden). A polystyrene tube filled with 0.35 mL of 1.7 mM ATP in 50 mM Tris-acetate buffer solution containing 10 mM $MgSO_4$ and 2 mM EDTA was supplied with 5 μL enzyme (~0.01 mg/mL) and placed into a sample compartment of the luminometer. The reaction was initiated by injecting 0.15 mL of 0.5 mM luciferin in the same buffer solution. Enzymatic activity was estimated by the maximum intensity of emitted light (flash) and expressed in relative light units (RLU/s). Unless stated otherwise, the activity was corrected for the spectral response of a photomultiplier in a FB12 luminometer based on the spectral parameters of the light emitted in the reaction.

The pH dependence of the enzymatic activity was measured under similar conditions. The pH of both substrate solutions (ATP and LH_2) were adjusted prior to the measurement (±0.01 pH). At the pH values below 7.0, MES buffer was used instead of Tris-acetate.

2.6. Kinetic parameters

The K_m values for LH_2 and ATP were determined from the bioluminescence activity assays performed as described above. The concentration of one substrate was maintained at saturation (1.2 mM ATP or 0.15 mM LH_2, respectively) and the concentration of the other substrate