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Point mutations in firefly luciferase C-domain demonstrate its significance in green color of bioluminescence

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ABSTRACT

Firefly luciferase is a two-domain enzyme that catalyzes the bioluminescent reaction of firefly luciferin oxidation. Color of the emitted light depends on the structure of the enzyme, yet the exact color-tuning mechanism remains unknown by now, and the role of the C-domain in it is rarely discussed, because a very few color-shifting mutations in the C-domain were described. Recently we reported a strong red-shifting mutation E457K in the C-domain; the bioluminescence spectra of this enzyme were independent of temperature or pH. In the present study we investigated the role of the residue E457 in the enzyme using the *Luciola mingrelica* luciferase with a thermostabilized N-domain as a parent enzyme for site-directed mutagenesis. We obtained a set of mutants and studied their catalytic properties, thermal stability and bioluminescence spectra. Experimental spectra were represented as a sum of two components (bioluminescence spectra of putative "red" and "green" emitters); λ_{max} of these components were constant for all the mutants, but the ratio of these emitters was defined by temperature and mutations in the C-domain. We suggest that each emitter is stabilized by a specific conformation of the active site; thus, enzymes with two forms of the active site coexist in the reactive media. The rigid structure of the C-domain is crucial for maintaining the conformation corresponding to the "green" emitter. We presume that the emitters are the keto- and enol forms of oxyluciferin.

1. Introduction

Firefly luciferases catalyze a bioluminescent reaction of the firefly luciferin oxidation by molecular oxygen in the presence of Mg^{2+} and ATP [1]. These enzymes have a vast field of application. They are employed in biosensors [2–4], immunoassays [5–7], reporter systems for nucleic acid amplification technologies [8], and detectors of protein–protein interactions [9,10]. They are frequently used as reporter genes [11], and various ATP assays [12] are based on the luciferase reaction.

The luciferase catalytic reaction proceeds in two steps: 1) formation of the luciferyl adenylate, 2) and its further oxidation to the electronically excited form of the oxyluciferin (LO*) [13]. The relaxation of LO* is accompanied by the emission of visible light (λ_{max} is in the range of 536–623 nm) [14] with an exceptionally high quantum yield [15].

The X-ray structure of the substrate-free luciferase from *Photinus pyralis* was determined in 1996 [16]. It demonstrated that the enzyme consists of two domains: a large N-domain (~4–436 residues) is connected to a small C-domain (440–544) by a flexible hinge. In the free enzyme, the domains are separated by a large cleft, and the residues on both sides of this cleft are highly conserved [16].

The structure of the enzyme in its closed form was obtained later, when *Luciola cruciata* luciferase was trapped at the adenylation step of the reaction by DLSA [17]. According to this structure, the C-domain rotates around the N-domain as the substrates bind to the active site and the cleft closes. The X-ray structure of another rotated form of luciferase was obtained in a recent study, in which the enzyme was trapped in the light-emitting conformation [18]. This study demonstrated that two steps of the catalytic process are coupled by ~140° rotation of the C-domain. The authors pointed out that the structure of the fully-rotated enzyme should be used for further studies on the firefly luciferase color-tuning mechanism [18].

The early models of the active site showed that though the substrate binding center is located in the N-domain, Lys529 (*P. pyralis*) of the C-domain is crucial for the formation of the active enzyme–substrate complex [19–21]. The importance of the C-domain for the coupling of two steps of the reaction [22] was proven by the fact that in the absence of the C-domain the luciferase retained ~0.03% of its activity [23]. By now two residues of the C-domain are known to directly participate in the catalytic process: Lys529 [24] and Lys443 [25] (*P. pyralis*) stabilize the reaction intermediates on adenylation and oxidative steps of the reaction, respectively [24,25]. These residues are located ~20 Å apart on the opposite faces of the C-domain, rotation of which allows them to enter the active site at the corresponding steps of the reaction.

The role of the C-domain in the color-tuning mechanism remains mostly uninvestigated. In the absence of the C-domain, the bioluminescence color is strongly red-shifted (λ_{max} 620 nm as compared to ~560

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nm of the wild-type enzyme) [23]. Few mutations in the C-domain were described: F465R [26] (*P. pyralis*), P452S [27] (*L. cruciata*), and the double mutation P451C–V469C (*P. pyralis*) [28,29]. The mutation F465R increased the thermal stability of the enzyme and its activity at pH 6.5; the bioluminescent spectrum was unaltered at pH 7.8, while at pH 6.5 a slight shoulder in the red region was observed [26]. The P452S mutation shifted the emission maximum of bioluminescence from 562 nm to 595 nm at pH 7.8; at pH 6.5, it remained unaltered [27]. The P451C–V469C mutations resulted in a formation of an additional S–S bond [28], which led to significant changes in bioluminescent spectra at 25 °C and 37 °C: the mutant enzyme exhibited a single peak in the red region of the spectrum at pH 7.8 (λ_{max} ~ 602 nm). This mutation also resulted in a two-fold decrease of the specific activity, a four-fold increase of K_m (LH_2), and approximately two-fold increase of thermal stability of the enzyme [29]. The effect of the mutation was attributed to the increase of the solvent accessibility of substrates [28]. Recently we reported a new strong red-shifting mutation E457K in the C-domain of *Luciola mingrelica* luciferase [30]; it reduced the enzymatic activity to ~40% and increased the K_m (ATP) by the factor of ~3.5.

In this study we investigated the role of the Glu457 residue in the C-domain of firefly luciferase by site-directed mutagenesis. We replaced the Glu457 residue by several residues with different properties such as length of the side-chain, charge, and hydrophobicity (E457V, E457D, E457Q, and E457K mutants). We also introduced the A534R mutation in an attempt to form an additional bond between the Glu457 residue and the α-helix on the opposite side of the C-domain by introducing the A534R mutation; the E457V/A534R mutant was produced to identify the effect of the A534R mutation in absence of the putative bond. We used the *L. mingrelica* luciferase with the stabilized N-domain (TS luciferase) [31] as a parent enzyme to lessen the possible negative impact of the mutations on the enzyme. We studied catalytic properties, thermal stability, and bioluminescent spectra of the enzymes at various temperatures. All of the introduced mutations were red shifting to various extents. We created computer models of the TS luciferase corresponding to the three different forms of the enzyme (open, half-rotated, and fully rotated) and used them to explain the effects of the mutations. The results of this study threw light on the role of the C-domain in maintaining green bioluminescence of the luciferase.

2. Material and methods

2.1. Materials

The following reagents were obtained from Sigma-Aldrich (St Louis, USA): Na-ATP (cat. no. A2383), bovine serum albumin, dithiotreitol (DTT), yeast extract (cat. no. Y-0500). D-luciferin was from Lumtek (Moscow, Russia). TaqSE DNA polymerase was from Sibenzyme (Novosibirsk, Russia); T4 DNA ligase was from Sileks (Moscow, Russia). The following reagents were purchased from the indicated sources: oligonucleotide primers (Sintol; Moscow, Russia), bactotryptone (Becton Dickinson; USA), and lactose monohydrate (Panreac; Montcada, Spain). Competent *Escherichia coli* cells were prepared and transformed according to the method developed by Tu and coworkers [32]. Other chemicals used were analytical or chemical grade. The solutions were prepared using MilliQ grade water (Millipore, France).

2.2. Site-directed mutagenesis

The mutant enzymes were prepared on the basis of TS luciferase by overlap extension PCR with forward and reverse mutagenic primers (see Table A). The presence of mutations was confirmed by sequencing. The sequencing was performed using an ABI PRISM® BigDye™ Terminator v.3.1 kit with the subsequent analysis of the products on an ABI PRISM 3730 Avant automatic DNA sequencer.

2.3. Expression and purification of luciferase

The TS luciferase and its mutants were cloned into the expression vector pETL7 (GenBank No. HQ007050) [33]. The pETL7 plasmid encodes the luciferase protein with two differences compared with the native enzyme: additional N-terminal sequence MASK- and the C-terminal AKM peptide changed to the SGPVEHHHHHH. Thus, all enzymes were expressed in the form of C-terminal His_6-tagged proteins; the expression was performed according to the lactose autoinduction protocol [34] using the *E. coli* BL21(DE3)CodonPlus cells as a host. The in vivo bioluminescence of the enzymes was observed by adding the cell suspension to the 0.5 mM luciferin solution in 0.1 M Na-citrate buffer, pH 5.0 (1:1 vol/vol). The enzymes were purified to homogeneity (>95% according to SDS-PAGE) on 1 ml NiIDA column (Amersham, Sweden) as described earlier [35]. Purified enzymes were stored at 4 °C in 20 mM sodium-phosphate buffer (pH 7.5) containing 500 mM NaCl, 300 mM imidazole, 2 mM EDTA, and 1 mM DTT.

In the process of purification, the yield of the enzymes was estimated as the amount of pure protein obtained from a fixed amount of growth medium. The enzyme concentration was determined by absorbance at 280 nm.

2.4. Fluorescence spectra

Intrinsic fluorescence of the enzymes was measured using an LS 50B spectrofluorometer (PerkinElmer, England) in "fluorescence" mode at a slit width of 10 nm. The spectra were obtained at 25 °C for 1×10^{-6} M luciferase solutions in 0.05 M Tris–acetate buffer (2 mM EDTA, 10 mM MgSO4, pH 7.8) in the range of 300–400 nm at the excitation wavelengths of 280 nm. Spectra were smoothed using Quadratic Golay–Savitzky filter.

2.5. Circular dichroism (CD) spectra

Far-UV (from 195 to 260 nm) CD spectra were recorded on a JASCO J-815 spectrophotometer for the solutions of 0.2 mg/ml luciferase in 50 mM phosphate buffer (pH 7.8) at 25 °C. Buffer exchange was performed using PD SpinTran G-25 columns (GE Healthcare) according to the manufacturer's instructions. The path length of the cell was 1 mm. The scan rate was 20 nm/min; the resulting spectra are the average of five measurements. The baseline was subtracted using the JASCO J-815 software; the spectra were smoothed using Quadratic Golay–Savitzky filter.

2.6. Bioluminescence spectra

Bioluminescence spectra were measured using an LS 50B spectrofluorometer (PerkinElmer, England) in "bioluminescence" mode at a slit width of 10 nm as described previously [35,36]. The enzyme was added to the solution, containing all other components of the reaction mix and preheated to the given temperature. The temperature of the cuvette was controlled during the measurement. Data were automatically corrected for the spectral response of the R928 photomultiplier tube (PMT) using the FL WinLab software. Generally, the spectra selected for the analysis were recorded when the decrease in intensity during the recording interval did not exceed 5%. Spectra were smoothed using Quadratic Golay–Savitzky filter in FL WinLab software.

2.7. Enzyme activity and kinetic parameters

Luciferase activity was determined on a FB12 luminometer (Zylux, USA) at 22 °C using the maximum intensity of light emitted during the enzymatic reaction at saturating concentrations of substrates. The cuvette contained 340 μl of 1.7 mM ATP solution in 0.05 M Tris–acetate buffer (2 mM EDTA, 10 mM $MgSO_4$, pH 7.8) and 5 μl of luciferase solution. The reaction was initiated by injection of 150 μl of 0.5 mM luciferin

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in the same buffer. Activity was corrected by the spectral response of the PMT and expressed in relative light units (RLU/s) of the luminometer.

The K_m values for LH_2 and ATP were determined from bioluminescence activity assays. The concentration of one substrate was maintained at saturation and the concentration of the other substrate was varied (0.012–1.2 mM ATP and 0.014–0.5 mM LH_2). Kinetic constants were calculated from Michaelis–Menten graph using non-linear regression in Origin 8.0 software (OriginLab, USA).

2.8. Kinetics of thermal inactivation

The solutions of 1×10^{-7} M luciferase were prepared in 50 mM Tris–acetate buffer (pH 7.8), containing 10 mM $MgSO_4$, 2 mM EDTA and 0.2 mg/ml BSA, and stored on ice. The initial enzymatic activity was measured 15 minutes after solution preparation.

Aliquots of 15 μl (50 °C, rapid inactivation) or 50 μl (42 °C, slow inactivation) were placed in 0.6-ml microtubes and incubated at corresponding temperature. At given times (each 10–30 min in case of slow inactivation; each 60–90 s in case of rapid inactivation) one of the microtubes was removed from thermostat and put on ice. The enzymatic activity in the microtube was measured ~15 minutes later. A neutral filter of ~0.14% transmittance (Photooptic-filters, Russia) was placed in the cuvette compartment of the luminometer to keep the bioluminescence intensity within the dynamic range.

2.9. Bioinformatics

Three different homology models of TS luciferase were constructed. The first model depicts free enzyme; the structure of free *P. pyralis* luciferase (PDB code: 1LCI) [16] was used as the template. The second model represents luciferase at adenylation step of the reaction; in this case the structures of *P. pyralis* and *L. cruciata* bound to DLSA (PDB codes: 4G36 [37] and 2D1S [17]) were the templates. The third one corresponds to luciferase at oxidation step; it was modeled using the structure of cross-linked *P. pyralis* luciferase in second catalytic conformation (PDB code: 4G37) [37].

Homology modeling was performed using Modeller 9.11 [38]; for each template the bunch of 250–300 structures was obtained. All structures were evaluated using the QMEAN server [39], and the best model of each bunch was chosen.

The UCSF Chimera package [40] was used for visualization of the models, the hydrogen bond prediction and the analysis of the possible impacts of point mutations. Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

Raytraced images were produced by POV-Ray (www.povray.org).

3. Results

3.1. Basis for mutagenesis

Currently, very few color-shifting mutations in the C-domain of firefly luciferase are known and described in the literature. For this reason, the strong red-shifting mutation E457K, which we discovered earlier by random mutagenesis of the C-domain of TS luciferase [30], was of great interest to us.

The analysis of multiple-sequence alignment emphasizes the significance of the Glu457 residue. It shows that Glu457 is almost absolutely conserved across all the diverse ANL superfamily of adenylating enzymes (acyl-CoA synthetases, NRPS adenylation domains, and luciferase). The analysis of the available experimentally determined structures of ANL enzymes shows that the side-chain of Glu457 forms a hydrogen bond with the backbone oxygen of another highly conserved residue—Val471. This bond connects the first α-helix (Glu457) and the following β-sheet (Val471) of the C-domain of all ANL enzymes.

On the basis of preliminary studies we presumed that the spectral change associated with the mutation E457K was caused by the introduction of non-compensated positive charge [30]. In order to verify this theory, we varied the properties of the side-chain in the position 457: the size of the amino acid residue, its polarity and charge. For this purpose, a set of mutants (E457K, E457V, E457Q, and E457D) was obtained on the basis of TS luciferase. The stabilization of N-domain makes TS enzyme more tolerant to the new mutations, while the C-domains of TS and WT *L. mingrelica* luciferases remain identical and can be compared. For instance, the introduction of the mutation E457K into the WT enzyme resulted in a significant drop in activity compared with the mutant E457K/TS.

In most ANL enzymes the first and the last α-helices of C-domain are connected by the salt bridge formed between Glu457 and Arg534. In certain cases (e.g. *Populus tomentosa* 4-coumarate-CoA ligase) two non-covalent bonds are formed between these residues. Though Arg534 (or Lys534) is also conserved in the ANL superfamily, it is absent in luciferases from firefly beetles, wherein this position is occupied by short, mostly non-polar amino acid residues.

In our previous study we introduced the mutation A534R in the TS form of *L. mingrelica* luciferase and compared in vivo properties of this mutant and the mutant E457V/A534R of the same enzyme [30]. Here we conducted more detailed studies of the mutants A534R and E457V/A534R.

Thus, the following set of mutants was obtained on the basis of TS luciferase: E457V, E457Q, E457D, A534R, and E457V/A534R. All the mutations were confirmed by sequencing.

3.2. Expression, purification, and structural analysis of the enzymes

As the enzymes were expressed, their in vivo bioluminescence was observed. Its color was red for mutants E457K, E457V, E457Q, and E457V/A534R, and orange for E457D and A534R. In the similar conditions the color of TS bioluminescence was orange-yellow. The light was very bright for all the mutants with the exception of E457D and A534R.

The yield of the enzymes also varied. While the mutations E457K and E457V had no noticeable effect on the protein yield, single mutations E457Q and E457D decreased it by a factor of two. The effect of the mutation A534R was even more severe: the yield decreased five-fold for both single (A534R) and double (E457V/A534R) mutants.

3.3. Kinetic properties of the mutants

We studied the following kinetic properties of the purified enzymes: specific activity, thermal stability, and K_m values for both substrates (ATP and luciferin).

3.3.1. Specific activity

The activity of the luciferases was characterized by the maximal intensity of the light emitted during the enzymatic reaction at saturating concentrations of substrates (flash height-based activity assay). Sensitivity of the 9078B photomultiplier tube (ET Enterprises Ltd, Uxbridge, UK) in FB12 luminometer strongly depends on the color of emitted light. We corrected the experimental data using spectral parameters for the enzymes, obtained in the course of this study, and the data on spectral response of the PMT of FB12 luminometer, provided by the manufacturer. Thus, resulting specific activities (Table 1) are compatible regardless of the spectral properties of the enzymes.

Single mutations E457Q and A534R had a negligible effect on enzymatic activity. Activity of the mutant E457V was slightly higher than that of TS enzyme. Meanwhile, combination of A534R and E457V mutations decreased luciferase activity approximately by the factor of two. The mutation E457K had a similar effect on the enzyme. The mutation E457D caused the most significant activity loss: the mutant possessed only 20% of the activity of TS luciferase.

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Table 1
Catalytic properties and thermal stability of the enzymes.

Enzyme	Relative specific activity, %	K_m, μM		Half-life (45 °C), min	Half-life (50 °C), min
		LH_2	ATP		
TS	100 ± 5	52 ± 5	26 ± 6	67 ± 6	4.6 ± 0.4
E457D	20 ± 2	25 ± 4	66 ± 7	96 ± 6	4.3 ± 0.3
A534R	100 ± 5	14 ± 4	27 ± 5	100 ± 9	8.5 ± 0.6
E457V	133 ± 6	16 ± 4	31 ± 4	44 ± 4	2.4 ± 0.2
E457Q	102 ± 5	17 ± 4	33 ± 4	45 ± 5	3.0 ± 0.3
E457V/A534R	47 ± 3	21 ± 4	94 ± 9	34 ± 3	2.3 ± 0.2
E457K	43 ± 3	46 ± 5	55 ± 6	34 ± 4	2.3 ± 0.2

3.3.2. Substrate affinity

We measured K_m values of the enzymes for both ATP and LH_2 (Table 1). Most of the mutations decreased K_m value for luciferin by the factor of two. The single exception is the mutant E457K: this mutation had no effect on luciferase affinity toward luciferin.

Yet the mutation E457K increased K_m value for ATP twofold; the mutation E457D had the same effect. The K_m values for ATP for the mutants E457V, E457Q, and A534R were almost the same as those for TS; the mutant E457V/A534R had the highest K_m among the enzymes.

3.3.3. Thermal stability

To estimate the impact of mutations on structural stability of TS luciferase we studied enzyme inactivation at 45 °C and 50 °C (Table 1). Most of the mutations had similar effect on the enzyme stability: it slightly decreased both at 45 °C and 50 °C. Surprisingly, E457D mutation increased thermal stability of the enzyme at 45 °C; at 50 °C stability of the enzyme was similar to that of TS. The enzyme with single A534R substitution had higher stability than the initial enzyme at all temperatures, which may be a result of the predicted H-bonds formation.

3.4. Evaluation of structural changes induced by the mutations

The mutations of the C-domain had a significant effect on thermal stability and activity of the TS luciferase. Theoretically, the mutations could affect the folding of the C-domain of the enzyme.

In order to evaluate the effect of the mutations on the enzyme structure, we studied the intrinsic fluorescence at the excitation wavelength of 280 nm for all the obtained mutants. All fluorescence spectra were identical (as an example, the spectra obtained for TS and the mutants with the most altered properties – E457D and E457K – are presented in Fig. A.1). However, the single Trp residue (Trp419) in the structure of the TS luciferase is located on the back of the N-domain far from both the active site and the residues E457 and A534 of the C-domain. Therefore, the similarity of the spectra only demonstrates that the mutations did not induce dramatic structural changes of the N-domain, yet cannot indicate the correct folding of the C-domain.

Far-UV CD spectra obtained for TS enzyme and the mutants E457K and E457D are shown in Fig. 1. The spectra of the mutants were somewhat altered; the effect of the mutations is most pronounced in the wavelength region of 195–200 nm. However, the analysis of the spectra showed that there were no pronounced differences between the percentages of secondary structure calculated for all the mutants. Therefore, the effect of the mutations cannot be attributed to the unfolding of the C-domain.

3.5. Bioluminescence spectra

The maxima and shape of firefly luciferase bioluminescence spectra are highly dependent on the reaction temperature. We analyzed bioluminescence spectra of TS luciferase and its mutant forms at three different temperatures: 10 °C, 25 °C, and 42°C (see Fig. 2). The most significant parameters of these spectra are summarized in Table 2.

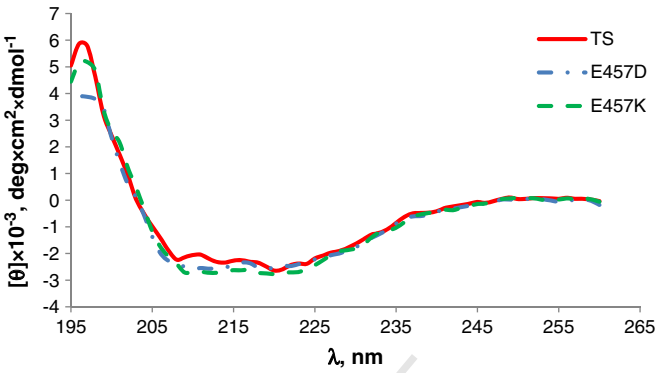

Fig. 1. Far-UV CD spectra of TS luciferase and its mutant forms (E457K and E457D) at 25 °C. The luciferase concentration was 0.2 mg/ml (50 mM phosphate buffer, pH 7.8). Each spectrum represents the average of three scans.

At high temperature (42 °C) all of the enzymes have the same λ_{max}, though the form of the spectrum (characterized by its half-width) varies. Spectral properties of TS enzyme strongly depend on the temperature: at 10 °C, λ_{max} is 41 nm lower than λ_{max} measured at 42 °C; at room temperature the λ_{max} is 565 nm, and the resulting bioluminescence color is yellow. In comparison with TS, all the mutants are red-shifted at room temperature. Yet they can be divided into three groups based on the way the reaction temperature defines their spectral properties.

The first group comprises the mutants E457D and A534R. In their case, the red shift is pronounced at 25 °C, whereas at lower and higher temperatures spectra of these mutants are similar to those of TS enzyme. Interestingly, the form of these spectra changes as well: at 25 °C they are noticeably wider than at other temperatures.

The second group comprises the mutants E457V, E457Q and E457V/A534R. These mutants are red-shifted at 10 °C and 25 °C, but the difference between λ_{max} values at 10 °C and 42 °C is no less than 27 nm. At 42 °C their bioluminescence spectra seem symmetrical, though at lower temperatures their shapes are complex.

The third group comprises only the mutant E457K, which is the strongly red-shifted. The spectral parameters of this mutant are virtually independent of temperature. The λ_{max} values at all temperatures are ~602 nm; all spectra have symmetric form and similar half-width.

Spectra for all the enzymes (except for the mutant E457K) are nearly monomodal only at 42 °C. We conducted curve fittings assuming Gaussian energy distribution functions to analyze experimental data obtained at lower temperatures. Usually three functions are used to reproduce experimental spectra [41,42], yet in our case two components were sufficient. Thus, most of the data on bioluminescent spectra were represented as a sum of two different components: the monomodal spectra of "green" and "red" emitters. At high temperature the "red" emitter prevails, but the ratio of "green" emitter increases along with a decrease in temperature. This tendency is most pronounced for the TS enzyme and the mutants E457D and A534R. We used Peak Analyzer tool provided by OriginPro 8 to analyze the experimental spectra and isolate their monomodal components (see Fig. A.2). The results of this analysis are summarized in Table 3. In a few cases we were unable to mathematically isolate peaks of the two components (E457K and E457Q at 25 °C) and analyzed the peaks as if they were monomodal. In these cases the corresponding data in Table 3 are absent and marked as N/A.

Though the parameters of hypothetical monomodal peaks were calculated independently for each experimental spectrum and were not predetermined, the location of the first peak (corresponding to the putative "green" emitter) is constant for all of the mutants. The λ_{max} of the second peak (corresponding to the putative "red" emitter) varies within a short range, which implies that this difference is determined merely by variation of the microenvironment of the emitter. The only changing

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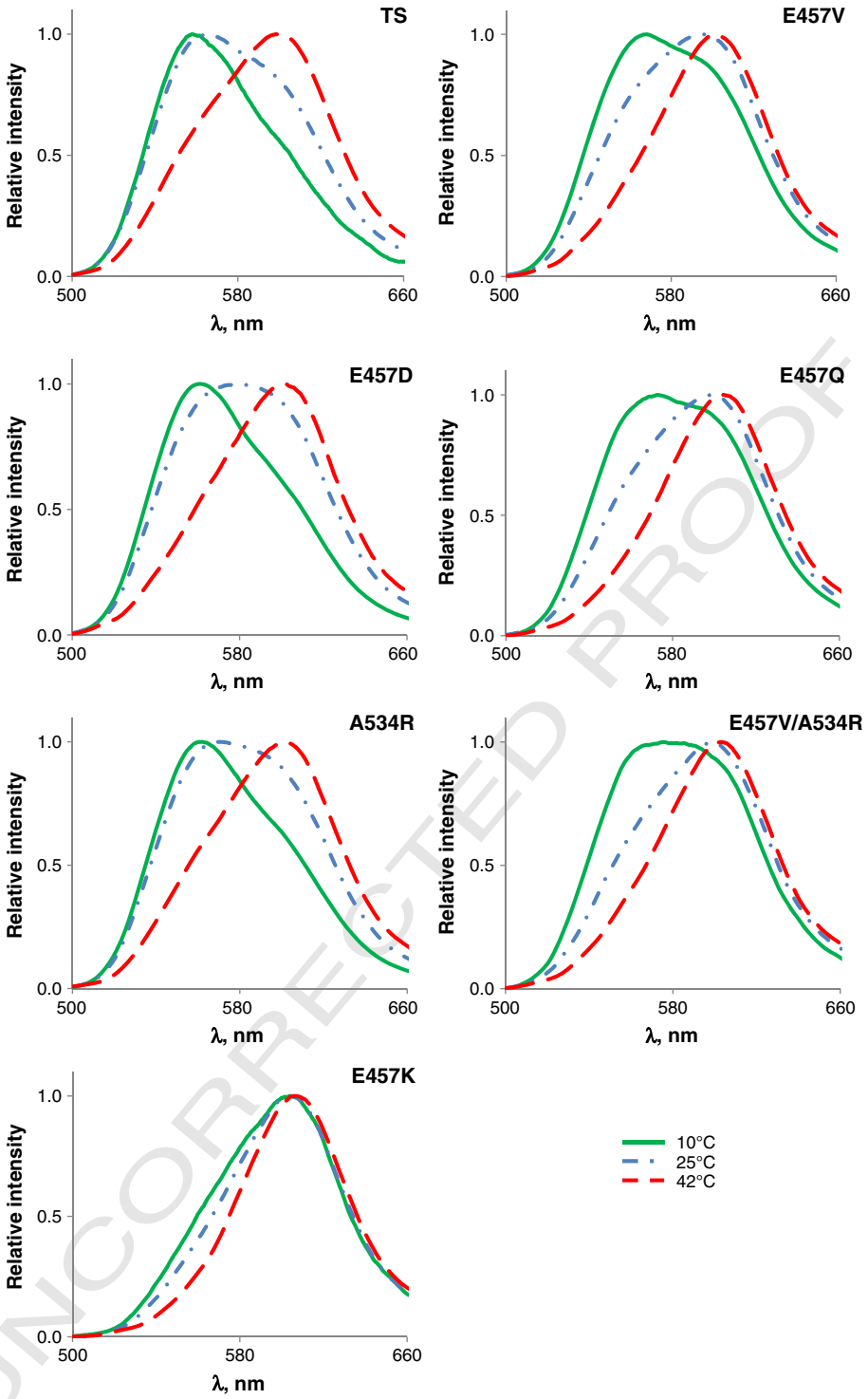

Fig. 2. Various types of temperature dependence of bioluminescent spectra of TS luciferase and its mutant forms (measured at pH 7.8; [luciferase] = 1 μM).

parameter is the intensity of two peaks: the intensity of "green" emitter increases along with the temperature decrease; the intensity of the red peak demonstrates the opposite trend. The only enzyme for which the amount of "green" emitter is negligible at any given temperature is the E457K mutant.

4. Discussion

The recognized function of the firefly luciferase C-domain is coupling adenylation and oxidative steps of the catalytic reaction [22]. Yet the C-domain remains largely unstudied, and the effects of mutations in this region are rather difficult to predict.

We focused our study on the role of the conserved residue Glu457: on the way its properties affect the structure of the C-domain and thus the whole enzyme. On the basis of the known X-ray structures of firefly luciferases, we produced three models of the TS luciferase in three different states: free form [16], half-rotated state [17,37] (adenylation step), and fully rotated state [37] (oxidation step). Using these models, we analyzed the impact of the mutations on the C-domain structure.

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Table 2
The features of bioluminescence spectra of TS luciferase and its mutant forms.*

Enzyme	λ_{max} (half-width), nm		
	10 °C	25 °C	42 °C
TS	558 (72)	565 (86)	599 (81)
E457D	562 (76)	578 (88)	602 (73)
A534R	562 (76)	571 (88)	601 (77)
E457V	568 (86)	594 (84)	602 (67)
E457Q	573 (87)	599 (82)	605 (65)
E457V/A534R	576 (87)	600 (79)	603 (66)
E457K	604 (74)	603 (68)	606 (61)

* Measured at pH 7.8; [luciferase] = 1 μM; measurement errors are ±5 nm for λ_{max}, ± 4 nm for the half-width.

The residue Glu457 is absolutely conserved in all luciferases. It is located in the α-helix and forms a conserved hydrogen bond with the main-chain of Val471 of the adjacent β-sheet (Fig. 3).

Interestingly, although the microenvironment of this residue is mildly affected by the C-domain rotation, this bond remains unaltered. In most of the ANL enzymes, Glu457 can also form an ionic bond with the side-chain of Arg534 [43–45] that is located in another α-helix on the opposite side of C-domain. However, in firefly luciferases position 534 is occupied by short, usually non-polar residues, such as Ala or Gly. The non-covalent bonds between the structural elements of the C-domain are rather scarce. Thus, the addition or disappearance of a non-covalent bond may have a significant impact on the enzyme stability.

The structural analysis of TS luciferase structure in its free form demonstrated that all mutations of Glu457 result in the loss of the Glu457–Val471 hydrogen bond. Indeed, most of the mutations destabilized the enzyme (see Table 1). The only exceptions were the mutant A534R, increased stability of which may indicate the formation of an additional ionic bond Glu457–Arg534 (Fig. 4), and the mutant E457D. The similarities of properties for the mutants E457D and A534R and the significantly decreased specific activity of the mutant E457D imply

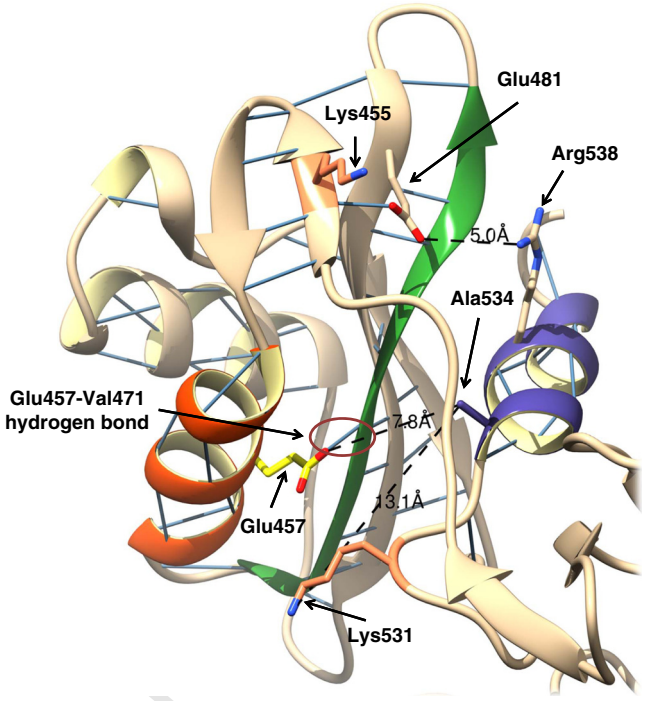

Fig. 3. The position of Glu457 and Ala534 residues in the C-domain structure of TS luciferase (free form). Lys455 and Lys531 belong to the active site. The model is based on the PDB structure 1LCI [14].

Table 3
Parameters of theoretical monomodal spectra, corresponding to the putative "green" and "red" emitters.[a]

pH 7.8	"Green" emitter		"Red" emitter	
	λ_{max}, nm	Area, % (percent of total peak area)	λ_{max}, nm	Area, % (percent of total peak area)
TS				
10°C	554	42	588	58
25°C	553	26	591	74
42°C	551	9	598	91
E457D				
10°C	554	38	589	62
25°C	553	19	592	81
42°C	–	–	597	100
A534R				
10°C	554	38	590	62
25°C	554	22	592	78
42°C	–	–	596	100
E457V				
10°C	554	24	591	76
25°C	552	11	594	89
42°C	–	–	599	100
E457Q				
10°C	554	21	593	79
25°C	552	9	596	91
42°C	–	–	602	100
E457V/A534R				
10°C	554	20	593	80
25°C	N/A	N/A	593	100
42°C	–	–	601	100
E457K				
10°C	554	7	601	93
25°C	N/A	N/A	601	100
42°C	–	–	606	100

[a] N/A stands for the data corresponding to the bioluminescent spectra, for which we were unable to mathematically isolate the peaks of the two components.

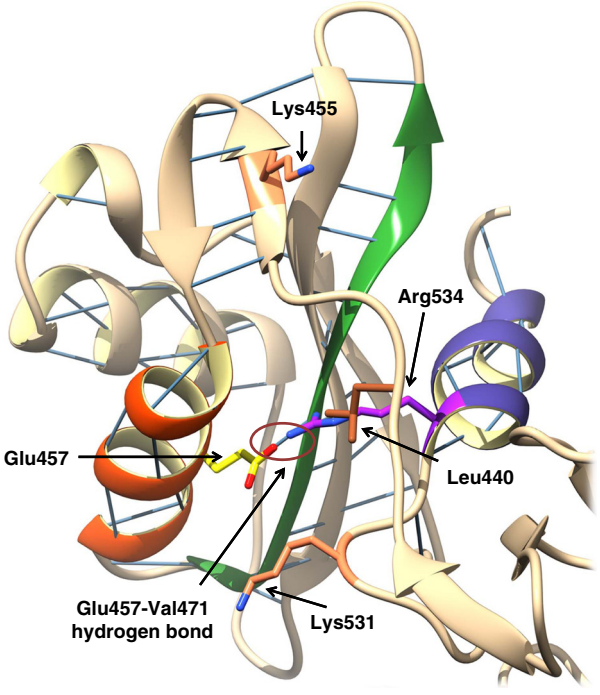

Fig. 4. The ionic bond between Glu457 and Arg534 residues in the C-domain structure of A534R mutant luciferase (free form). Lys455 and Lys531 belong to the active site. The model is based on the PDB structure 1LCI [14].

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that the structure of the mutant E457D was considerably altered by the mutation; indeed, the side-chain of Asp457 clashes into the nearby residues in all of its rotameric forms. We were unable to model the resulting changes, yet we presume the formation of an extra bond somewhat similar to the E457-R534 bond, which may be formed in the mutant A534R. For instance, the ionic bond might be formed between Arg538 and Glu481 residues (Fig. 3); such bond may fix mutual position of the β-sheet (Glu481) and α-helix (Arg538). In the molecule of TS luciferase, these residues are located ~5 Å from each other; however, in the mutant E457D, this distance could possibly be reduced.

The catalytic parameters of the TS enzyme were also altered by the mutations. Glu457 mutations decreased the K_m for LH_2; the only exception was the mutant E457K (Table 1). This effect can also be attributed to the loss of the E457–V471 hydrogen bond, which the mutants are unable to form even in the half-rotated conformation of the enzyme. Thus, the weakened contact between the α-helix and the β-sheet of the C-domain results in the increase of luciferase affinity to luciferin.

The value of K_m for LH_2 for A534R is also very low, while the K_m for ATP remains unaltered by the mutation (Table 1). The origin of the effect on K_m for LH_2 is ambiguous. The computer modeling shows that the E457–R534 bond is only favorable for the free form of the enzyme, while the rotation of the C-domain creates steric hindrances in this region. By the final step of the catalytic reaction, this bond has to be broken to relieve the steric pressure. The E457–R534 bond probably binds the two α-sheets in the closed form of the enzyme, but the Arg534 residue may actually push them apart as the substrates bind and the conformation of the enzyme changes. In this case, the effect of the A534R mutation on the K_m for LH_2 may be similar to that of the absence of the E457–V471 hydrogen bond: the connection between the structural elements in the region actually weakens as the reaction proceeds. This may be the reason why, as opposed to other ANL enzymes, the Arg residue was evolutionary removed from firefly luciferases.

The double mutation E457V/A534R affects the enzyme properties in somewhat unexpected way. The K_m (LH_2) value of this enzyme correlates with the K_m (LH_2) of E457V and A534R mutants, yet the K_m (ATP) value is greatly increased (Table 1). Its bioluminescent properties also differ from those of the corresponding single mutants (Fig. 2). Structural analysis demonstrates that, in the absence of Glu457, the side-chain of Arg534 is most likely turned in the direction of the C- and N-domain interface (Fig. 5). The C-domain rotation alters the microenvironment of the residue Arg534, bringing it closer to the catalytic residue Lys531 (Lys529 in *P. pyralis*) that plays essential role at the adenylation step of the reaction [24]. The uncompensated positive charge on the Arg534 residue seems to affect the affinity of the enzyme toward the ATP; the long side-chain also creates steric hindrance as the C-domain rotates.

The increase of K_m (ATP) value was also observed for the E457D and E457K mutants (Table 1). In the first case, this effect may be explained by the structural changes caused by steric clashes. The changes induced by the E457D mutation stabilize the free form of the enzyme (the thermal inactivation is performed in the absence of substrates). However, the change of the C-domain conformation is required for the catalytic reaction to proceed. The experimental data indicate that the transition from the free form to the half-rotated and fully rotated forms of the molecule may be hindered by the mutation E457D. This may explain the substantial drop of the specific activity of the enzyme and the increase of K_m (ATP) observed along with the stabilization of the enzyme.

The Lys457 residue of E457K mutant is directed toward the interface between the domains. We assumed previously [30] that the positive charge of the residue Lys457 may be the reason for the increase of K_m for ATP. Moreover, according to the computer analysis, the long side-chain of Lys457 residue causes steric pressure in the molecule of the enzyme. The positive charge on the Lys residue may also be the reason for an increased (as compared to the other E457 mutants) K_m (LH_2) value. Thus, as opposed to any other E457 mutations, this residue is long and charged. While the direct electrostatic influence of Lys457 on the binding sites is unlikely, the induced indirect interactions could affect them and, by this, the state of the emitter.

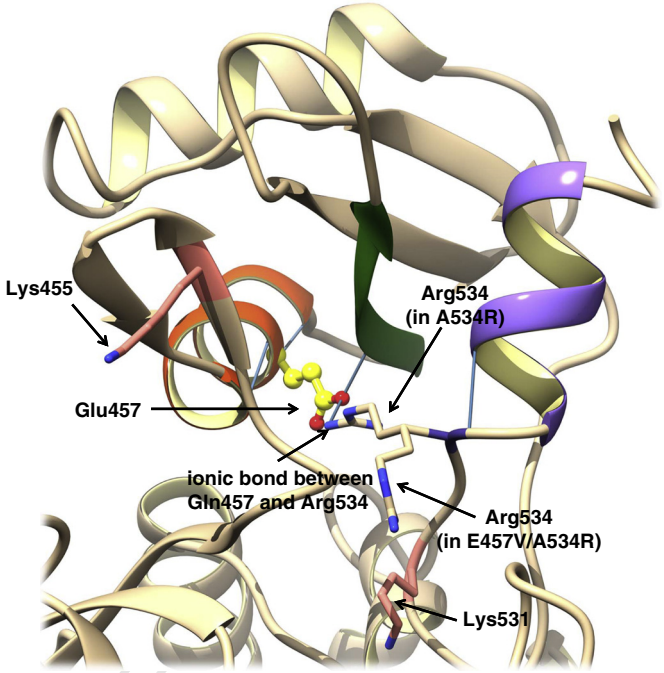

Fig. 5. The positions of Arg534 residue in the C-domain structure of A534R and E457V/A534R mutants of TS luciferase (half-rotated form). Residues Lys455 and Lys531 belong to the active site. The model is based on the PDB structures 2D1S [17] and 4G36 [37].

Thus, all the mutations of the residue Glu457 seem to result in a loss of conserved hydrogen bond between its secondary structure elements. In certain cases these mutations also result in serious steric hindrances (E457D) and the unfavorable presence of uncompensated positive charge (E457V/A534R). The A534R mutation stabilizes the enzyme, and this effect is possibly associated with the formation of the additional Glu457-Arg534 ionic bond. However, this effect seems to be diminished by the steric hindrance that increases as the enzyme rotates; at the final step of the reaction, this bond has to be broken. All in all, the mutations of these regions of the C-domain result in weakening the connections between the structural elements of the C-domain and by that in making the C-domain less rigid.

The color-tuning mechanism of firefly luciferases remains a mystery; it may depend on various factors such as the active forms of oxyluciferin [42], differences in polarization of its microenvironment [46], solvent accessibility of the emitter [47], and size of the cavity of luciferase [47]. Many studies were devoted to the impact of the N-domain residues on the color of bioluminescence which is extensively studied, yet the role of the C-domain is generally omitted.

The bioluminescent spectrum of firefly luciferase has a complex shape and can be represented as a sum of two monomodal spectra corresponding to the "red" and "green" emitter; their ratio defines the resulting form of experimental spectrum and its emission maximum [47]. This ratio depends on various parameters, including the temperature.

Our data demonstrate that the amount of the "green" emitter decreases with the temperature increase (see Table 3); it is equally true for the TS enzyme and all of its mutants with the exception of the enzyme E457K, which has a monomodal spectrum with no temperature dependence. We presume that each emitter requires its own type of microenvironment of the active site. The green emission is stabilized by rigid and non-polar microenvironment [17]; the more loosened conformation allows greater energy loss resulting in red emission.

All mutants are red-shifting, though the effect is pronounced to a different extent depending on the mutation (Fig. 2). The patterns of temperature dependence also varied: the mutants A345R and the

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E457D were similar to the TS luciferase, though the amount of "green" emitter was somewhat lower at 10–25 °C and negligibly low at 42 °C; the mutant E457K nearly lacked the "green" emitter at all temperatures; the bioluminescence spectra of other mutants were largely defined by the "red" emitter at all temperatures, though at 10 °C a noticeable amount of "green" emitter remained in the system (Fig. A.2).

Interestingly, the emission maxima of the putative "green" and "red" emitters of the TS luciferase were almost equal to those of all the C-domain mutants at all temperatures. This result implies that the nature of emitters is essentially the same for all the mutants, which requires two distinctive forms of the active site to stabilize each of them.

The previous studies of fluorescence for oxyluciferin and dimethyloxyluciferin demonstrated that the light emission at ~ 550 nm corresponds to the enol/enolate-oxyluciferin, while its keto-form emits light at ~602 nm as proven by the data on dimethyloxyluciferin which is constrained to exist in keto form [48]. Our data agree with these results. The theory of keto–enol tautomerism as a color-tuning mechanism of firefly luciferase was left out of account for a while, yet there is strong evidence that it is worth of attention [49].

We suggest that at decreased temperatures two forms of firefly luciferase coexist in reaction media. These forms differ by the conformations of their active sites: one of them stabilizes oxyluciferin in its enol/enolate form, while the other one stabilizes its keto-form. As the temperature increases the conformation, corresponding to the keto-form, becomes predominant. The mutations of the C-domain decrease the stability of the "green" form of luciferase; this effect is pronounced to a variable degree, so that the bioluminescence spectra of E457D and A534R are almost identical to that of the TS luciferase, while in E457K the "green" form is completely destabilized. Thus, the intact structure of the C-domain is required for maintaining the green bioluminescence of firefly luciferases.

5. Conclusions

We demonstrate that the slight changes in the highly conserved regions of C-domain weaken the connections between its secondary structure elements and by that change the spectrum of bioluminescence, shifting it to the red region. This shift corresponds to the changing ratio of "green" and "red" emitters in the reaction media. However, the mutations do not affect the nature of the emitters.

We suggest that two forms of the enzyme coexist at the final step of the reaction; one of them stabilizes the "green" emitter; the other, "red." The similarity of calculated emission maximums of both emitters agrees with the results obtained for the emission of keto- and enol forms of the oxyluciferin. Thus, the intact structure of the C-domain may be essential for maintaining the rigid and non-polar state of the active site, which is required for the stabilization of oxyluciferin in its enol/enolate form.

These results support the theory of keto–enol tautomerism of oxyluciferin as a color-tuning mechanism of firefly luciferases.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbapap.2014.04.021.

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References

[1] M. Deluca, Firefly luciferase, Adv. Enzymol. Relat. Areas Mol. Biol. 44 (1976) 37–68.
[2] F. Ataei, M. Torkzadeh-Mahani, S. Hosseinkhani, A novel luminescent biosensor for rapid monitoring of IP3 by split-luciferase complementary assay, Biosens. Bioelectron. 41 (2013) 642–648.
[3] C.I. Stains, J.L. Furman, J.R. Porter, S. Rajagopal, Y. Li, R.T. Wyatt, I. Ghosh, A general approach for receptor and antibody-targeted detection of native proteins utilizing split-luciferase reassembly, ACS Chem. Biol. 5 (2010) 943–952.
[4] R. Cheraghi, S. Hosseinkhani, J. Davoodi, M. Nazari, Z. Amini-Bayat, H. Karimi, M. Shamseddin, F. Gheidari, Structural and functional effects of circular permutation on firefly luciferase: in vitro assay of caspase 3/7, Int. J. Biol. Macromol. 58 (2013) 336–342.
[5] M. Maeda, New label enzymes for bioluminescent enzyme immunoassay, J. Pharm. Biomed. Anal. 30 (2003) 1725–1734.
[6] F. Akter, M. Mie, E. Kobatake, Aptamer-based protein detection using a bioluminescent fusion protein, Analyst 137 (2012) 5297–5301.
[7] Y. Yamakawa, H. Ueda, A. Kitayama, T. Nagamune, Rapid homogeneous immunoassay of peptides based on bioluminescence resonance energy transfer from firefly luciferase, J. Biosci. Bioeng. 93 (2002) 537–542.
[8] O.A. Gandelman, V.L. Church, C.A. Moore, G. Kiddle, C.A. Carne, S. Parmar, H. Jalal, L.C. Tisi, J.A.H. Murray, Novel bioluminescent quantitative detection of nucleic acid amplification in real-time, PLoS One 5 (2010) e14155.
[9] H.-K. Kim, E.J. Cho, S. mi Jo, B.R. Sung, S. Lee, S.-H. Yun, A split luciferase complementation assay for studying in vivo protein–protein interactions in filamentous ascomycetes, Curr. Genet. 58 (2012) 179–189.
[10] T. Ozawa, A. Kaihara, M. Sato, K. Tachihara, Y. Umezawa, Split luciferase as an optical probe for detecting protein–protein interactions in mammalian cells based on protein splicing, Anal. Chem. 73 (2001) 2516–2521.
[11] J. Brogan, F. Li, W. Li, Z. He, Q. Huang, C.-Y. Li, Imaging molecular pathways: reporter genes, Radiat. Res. 177 (2012) 508–513.
[12] A. Lundin, Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites, Methods Enzymol. 305 (2000) 346–370.
[13] A. Green, W.D. McElroy, Function of adenosine triphosphate in the activation of luciferin, Arch. Biochem. Biophys. 64 (1956) 257–271.
[14] V.R. Viviani, The origin, diversity, and structure function relationships of insect luciferases, Cell. Mol. Life Sci. 59 (2002) 1833–1850.
[15] K. Niwa, Y. Ichino, S. Kumata, Y. Nakajima, Y. Hiraishi, D. Kato, V.R. Viviani, Y. Ohmiya, C.D.S. Ufscar, Quantum yields and kinetics of the firefly bioluminescence reaction of beetle luciferases, Photochem. Photobiol. 86 (2010) 1046–1049.
[16] E. Conti, N.P. Franks, P. Brick, Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes, Structure 4 (1996) 287–298.
[17] T. Nakatsu, S. Ichiyama, J. Hiratake, A. Saldanha, N. Kobashi, K. Sakata, H. Kato, Structural basis for the spectral difference in luciferase bioluminescence, Nature 440 (2006) 372–376.
[18] B.R. Branchini, J.C. Rosenberg, D.M. Fontaine, T.L. Southworth, C.E. Behney, L. Uzasci, Bioluminescence is produced from a trapped firefly luciferase conformation predicted by the domain alternation mechanism, J. Am. Chem. Soc. 133 (2011) 11088–11091.
[19] T.P. Sandalova, N.N. Ugarova, Model of the active site of firefly luciferase, Biochem. Mosc. 64 (1999) 962–967.
[20] B.R. Branchini, R.a. Magyar, M.H. Murtiashaw, S.M. Anderson, M. Zimmer, Site-directed mutagenesis of histidine 245 in firefly luciferase: a proposed model of the active site, Biochemistry 37 (1998) 15311–15319.
[21] N.P. Franks, A. Jenkins, E. Conti, W.R. Lieb, P. Brick, Structural basis for the inhibition of firefly luciferase by a general anesthetic, Biophys. J. 75 (1998) 2205–2211.
[22] K. Ayabe, T. Zako, H. Ueda, The role of firefly luciferase C-terminal domain in efficient coupling of adenylation and oxidative steps, FEBS Lett. 579 (2005) 4389–4394.
[23] T. Zako, K. Ayabe, T. Aburatani, N. Kamiya, A. Kitayama, H. Ueda, T. Nagamune, Luminescent and substrate binding activities of firefly luciferase N-terminal domain, Biochim. Biophys. Acta Proteins Proteomics 1649 (2003) 183–189.
[24] B.R. Branchini, M.H. Murtiashaw, R.a. Magyar, S.M. Anderson, The role of lysine 529, a conserved residue of the acyl-adenylate-forming enzyme superfamily, in firefly luciferase, Biochemistry 39 (2000) 5433–5440.
[25] B.R. Branchini, T.L. Southworth, M.H. Murtiashaw, S.R. Wilkinson, N.F. Khattak, J.C. Rosenberg, M. Zimmer, Mutagenesis evidence that the partial reactions of firefly bioluminescence are catalyzed by different conformations of the luciferase C-terminal domain, Biochemistry 44 (2005) 1385–1393.
[26] G.H.E. Law, O.a. Gandelman, L.C. Tisi, C.R. Lowe, J.A.H. Murray, Mutagenesis of solvent-exposed amino acids in *Photinus pyralis* luciferase improves thermostability and pH-tolerance, Biochem. J. 397 (2006) 305–312.
[27] N. Kajiyama, E. Nakano, Isolation and characterization of mutants of firefly luciferase which produce different colors of light, Protein Eng. 4 (1991) 691–693.
[28] M. Nazari, S. Hosseinkhani, L. Hassani, Step-wise addition of disulfide bridge in firefly luciferase controls color shift through a flexible loop: a thermodynamic perspective, Photochem. Photobiol. Sci. 12 (2013) 298–308.
[29] M. Nazari, S. Hosseinkhani, Design of disulfide bridge as an alternative mechanism for color shift in firefly luciferase and development of secreted luciferase, Photochem. Photobiol. Sci. 10 (2011) 1203–1215.
[30] M.I. Koksharov, N.N. Ugarova, Strategy of mutual compensation of green and red mutants of firefly luciferase identifies a mutation of the highly conservative residue E457 with a strong red shift of bioluminescence, Photochem. Photobiol. Sci. 12 (2013) 2016–2027.
[31] M.I. Koksharov, N.N. Ugarova, Thermostabilization of firefly luciferase by in vivo directed evolution, Protein Eng. Des. Sel. 24 (2011) 835–844.
[32] Z. Tu, G. He, K.X. Li, M.J. Chen, J. Chang, L. Chen, Q. Yao, D.P. Liu, H. Ye, J. Shi, X. Wu, An improved system for competent cell preparation and high efficiency plasmid transformation using different *Escherichia coli* strains, Electron. J. Biotechnol. 8 (2005).
[33] M.I. Koksharov, N.N. Ugarova, Triple substitution G216N/A217L/S398M leads to the active and thermostable *Luciola mingrelica* firefly luciferase, Photochem. Photobiol. Sci. 10 (2011) 931–938.
[34] F.W. Studier, Protein production by auto-induction in high-density shaking cultures, Protein Expr. Purif. 41 (2005) 207–234.
[35] Y.A. Modestova, G.Y. Lomakina, N.N. Ugarova, Site-directed mutagenesis of cysteine residues of *Luciola mingrelica* firefly luciferase, Biochem. Mosc. 76 (2011) 1147–1154.

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[36] M.I. Koksharov, N.N. Ugarova, Random mutagenesis of *Luciola mingrelica* firefly luciferase mutant enzymes with bioluminescence spectra showing low pH sensitivity, Biochem. Mosc. 73 (2008) 862–869.
[37] J.A. Sundlov, D.M. Fontaine, T.L. Southworth, B.R. Branchini, A.M. Gulick, Crystal structure of firefly luciferase in a second catalytic conformation supports a domain alternation mechanism, Biochemistry 51 (2012) 6493–6495.
[38] N. Eswar, B. Webb, M.a. Marti-Renom, M.S. Madhusudhan, D. Eramian, M.-Y. Shen, U. Pieper, A. Sali, Comparative protein structure modeling using MODELLER, Curr. Protoc. Protein Sci. (2007) (Chapter 2, Unit 2.9).
[39] P. Benkert, M. Künzli, T. Schwede, QMEAN server for protein model quality estimation, Nucleic Acids Res. 37 (2009) W510–W514.
[40] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, UCSF chimera—a visualization system for exploratory research and analysis, J. Comput. Chem. 25 (2004) 1605–1612.
[41] Y. Wang, H. Akiyama, K. Terakado, T. Nakatsu, Impact of site-directed mutant luciferase on quantitative green and orange/red emission intensities in firefly bioluminescence, Sci. Rep. 3 (2013) 2490.
[42] N.N. Ugarova, L.G. Maloshenok, I.V. Uporov, M.I. Koksharov, Bioluminescence spectra of native and mutant firefly luciferases as a function of pH, Biochem. Mosc. 70 (2005) 1262–1267.
[43] J. Shockey, J. Browse, Genome-level and biochemical diversity of the acyl-activating enzyme superfamily in plants, Plant J. 66 (2011) 143–160.
[44] P.A. Watkins, D. Maiguel, Z. Jia, J. Pevsner, Evidence for 26 distinct acyl-coenzyme A synthetase genes in the human genome, J. Lipid Res. 48 (2007) 2736–2750.
[45] A.M. Gulick, Conformational dynamics in the Acyl-CoA synthetases, adenylation domains of non-ribosomal peptide synthetases, and firefly luciferase, ACS Chem. Biol. 4 (2009) 811–827.
[46] N.N. Ugarova, L.Y. Brovko, Protein structure and bioluminescent spectra for firefly bioluminescence, Luminescence 17 (2002) 321–330.
[47] S. Hosseinkhani, Molecular enigma of multicolor bioluminescence of firefly luciferase, Cell. Mol. Life Sci. 68 (2011) 1167–1182.
[48] O.V. Leont'eva, T.N. Vlasova, N.N. Ugarova, Dimethyl- and monomethyloxyluciferins as analogs of the product of the bioluminescence reaction catalyzed by firefly luciferase, Biochemistry 71 (2006) 51–55.
[49] L.P. da Silva, R. Simkovitch, D. Huppert, J.C.G.E. da Silva, Oxyluciferin photoacidity: the missing element for solving the keto–enol mystery? ChemPhysChem 14 (2013) 3441–3446

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