ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 10, pp. 1147-1154. © Pleiades Publishing, Ltd., 2011.
Published in Russian in Biokhimiya, 2011, Vol. 76, No. 10, pp. 1407-1415.

Site-Directed Mutagenesis of Cysteine Residues of *Luciola mingrelica* Firefly Luciferase

Yu. A. Modestova, G. Yu. Lomakina, and N. N. Ugarova*

Faculty of Chemistry, Lomonosov Moscow State University, 119991 Moscow, Russia;
fax: (495) 939-2660; E-mail: nugarova@gmail.com

Received February 7, 2011
Revision received April 4, 2011

Abstract—Single mutants (C62S, C62V, C86S, C146S, C164S), double mutants (C62/146S, C62/164S, C86/146S, C146/164S), and triple mutant C62/146/164S of the *Luciola mingrelica* firefly luciferase carrying C-terminal His_6-tag were obtained on the basis of plasmid pETL7 by site-directed mutagenesis. Bioluminescence and fluorescence spectra were not altered by the introduced mutations. In the case of mutants C86S, C86/146S, C62/164S, and the triple mutant C62/146/164S, the K_m^{ATP} and $K_m^{LH_2}$ values were increased by a factor of ~1.5-1.9. Their expression level, specific activity, and thermal stability were significantly decreased. The other mutations had almost no effect on the K_m^{ATP} and $K_m^{LH_2}$ values, specific activity, and thermal stability of the enzyme. Thermal stability of the C146S mutant was increased by a factor of ~2 and 1.3 at 37 and 42°C, respectively. The possible mechanism of the influence of these mutations on properties and structure of the enzyme is discussed.

DOI: 10.1134/S0006297911100087

Key words: firefly luciferase, *Luciola mingrelica*, site-directed mutagenesis, cysteine residues, polyhistidine tag, kinetic parameters, thermal stability

Firefly luciferase (EC 1.13.12.7) catalyzes the air oxygen oxidation of firefly luciferin in the presence of Mg^{2+} and ATP [1]. Due to its high specificity and high quantum yield of the bioluminescence, firefly luciferase is widely used in bioanalytical applications [2]. This motivates continuing scientific interest in the investigation of firefly luciferase structure and functions [3]. Primary structures of more than 20 firefly luciferases of various genera and species are currently known. The firefly luciferase molecule contains free cysteine residues. The number of cysteine residues varies over a wide range (from 4 to 14 residues) depending on the firefly species. Though the cysteine residues do not belong to the active site, their mutagenesis was shown to affect activity and stability of luciferases [4, 5]. The role of these residues in the functioning of luciferase remains unclear. The most known *Photinus pyralis* luciferase from American fireflies contains four cysteine residues, three of which (81, 258, and 391) are conserved in most luciferases. Single substitutions of cysteine residues for serine using site-specific mutagenesis resulted in a 1.2-1.5-fold decrease in enzyme activity. Multiple substitutions of cysteine residues resulted in a more drastic activity decrease of *P. pyralis* luciferase. For instance, the mutant with four substitutions C81/216/258/391S maintained only 6.5% of its activity. Moreover, mutual influence of the mutations was clearly observed [4].

The *Luciola mingrelica* firefly luciferase contains eight cysteine residues, three of which correspond to the conservative cysteine residues of *P. pyralis* firefly luciferase – 82, 260, and 393. Mutant forms of *L. mingrelica* luciferase containing single substitutions of these cysteine residues for alanine were obtained previously [5]. These substitutions had no effect on bioluminescent and fluorescent spectra of the enzyme, though they led to a certain decrease of firefly luciferase binding constants for luciferin and ATP. The C393A mutant stability at 5-35°C was twofold higher than that of the WT (initial recombinant luciferase) enzyme. The C82A and C393A substitutions had no effect on the thermal stability of the enzyme. We previously used the pLR plasmid encoding firefly luciferase with structure identical to that of the native enzyme for the preparation of the enzyme mutant forms with single substitutions of the non-conserved cysteine

Abbreviations: DTT, dithiothreitol; k_{in}, inactivation rate constant; λ_{ex}, fluorescence excitation wavelength; LH_2, firefly luciferin; PCR, polymerase chain reaction; RLU, relative light units; WT, initial recombinant luciferase.

* To whom correspondence should be addressed.

residues C62S, C146S [6], and C164S [7]. These substitutions also had no significant effect on the catalytic and the spectral properties of the luciferase, but they resulted in an increase of the enzyme thermal stability and a decrease of inactivation rate constant dependence on the enzyme concentration (unlike the WT enzyme). Moreover, the influence of dithiothreitol (DTT) on luciferase stability was diminished. These effects were most pronounced for enzyme with the C146S substitution. Purification of recombinant luciferase obtained using the pLR plasmid is a complicated multistage process. To simplify the preparation process in this work, we used the recombinant *L. mingrelica* luciferase with C-terminal His_6-tag that was expressed in *Escherichia coli* cells transformed by the pETL7 plasmid [8]. This approach allowed us to simplify the scheme of enzyme purification and to increase luciferase yield due to the use of the highly effective expression system of the pET strain. The influence of the polyhistidine tag on the properties of the luciferase was not considered in the literature previously, but certain publications indicate that the introduction of a His_6-tag may alter properties of enzymes [9-13].

The goal of this study was to estimate the role of nonconserved cysteine residues in the *L. mingrelica* firefly luciferase molecule and to reveal the mutual influence of these residues and the effect of His_6-tag on the enzyme activity and thermal stability.

MATERIALS AND METHODS

The following reagents were used in the present work: restrictases *Bam*HI, *Bpu*14I, and TaqSE polymerase (SibEnzyme, Russia); oligonucleotides (Syntol, Russia); ATP disodium salt and BSA (Sigma, USA); D-luciferin (Lumtek, Russia). Other chemicals used were analytical or chemical grade. The solutions were prepared using Milli-Q grade water (Millipore, France).

DNA was sequenced 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.

Site-directed mutagenesis was performed in the region between *Nhe*I and *Bam*HI restriction sites (680 bp) of the *L. mingrelica* luciferase gene. Forward and reverse primers were respectively: 5′-ATTATAGGAGGCTAGCAAAATGG-3′ and 5′-GTAAATTGGATCCTTAGCGTG-3′. A set of partially overlapping primers encoding the substitution being introduced into the gene was also used (Table 1). The pETL7 plasmid was used as a template for the preparation of single mutants [8]. Multiple mutants were obtained using the respective mutant plasmids prepared on the basis of pETL7. In the first stage two fragments of the DNA encoding a mutation and having partially overlapping ends were synthesized. The reaction mix (50 μl) for the preparation of 5′-end fragment DNA contained 60 mM Tris-HCl (pH 8.5 at 25°C), 1.5 mM $MgCl_2$, 25 mM KCl, 10 mM 2-mercaptoethanol, 0.1% Triton X-100, 20 pmol of the forward primer, 20 pmol of the reverse primer carrying a substitution, 50 ng of the plasmid, 100 μg BSA, 50 μM dNTP, and 2.5 units of TaqSE DNA polymerase. The reaction mix (50 μl) for the preparation of 3′-end fragment had the same composition except for the presence of 20 pmol of the reverse primer and 20 pmol of the forward primer carrying the substitution. Polymerase chain reaction (PCR) was performed on a Tercik amplifier (DNA Technology, Russia) under the following conditions: 95°C, 1 min; 25 cycles for 1 min at

Table 1. Primers for introduction of point mutations

Substitution	Primer	T_a, °C
C62S	forward: 5'-GATATTACA**TCT**CGTTTAGCTGAGGCCATG-3'	60
	reverse: 5'-GCTAAACG**AGA**TGTAATATCAAAGTATTC-3'	54
C62V	forward: 5'-GATATTACAGTTC**GTT**TAGCTGAGGCCATG-3'	60
	reverse: 5'-CTTATGA**AAC**TATAATGTCAAGCAAATCG-3'	54
C86S	forward: 5'-AAAT**TCT**TCAGAATTTTCACTGCACAAAGC-3'	54
	reverse: 5'-GCAGTGAAAATGA**AGA**ATTTTTCATCCCTG-3'	52
C146S	forward: 5'-AAACAGTTACA**TCC**ATCAAAAAAATTGTTATTTTAG-3'	56
	reverse: 5'-TTTTTTTGAT**GGA**TGTAACTGTTTTTTGCACTTC-3'	56
C164S	forward: 5'-CCACGAT**TCT**ATGGAAACTTTTATTAAG-3'	54
	reverse: 5'-GTTTCCAT**AGA**ATCGTGGCCCCCAAAG-3'	61

Note: T_a, amplification temperature.

94°C, 1 min at T_a (Table 1), 2 min at 72°C; then completion for 10 min at 72°C. PCR products were purified by electrophoresis on 1% agarose gel with subsequent isolation from the gel using a Qiagen (USA) kit. The mutated PCR fragments were ligated by PCR under the aforementioned conditions (T_a = 53°C). The reaction mix (50 μl) was of the same composition but contained 5 ng of each fragment obtained at the previous stages in place of 50 ng of the initial plasmid. The reaction product was purified and isolated as described above. It was treated with restrictases *Bam*HI and *Bpu*14I (*Bpu*14I restriction site is located at the distance of 138 bp from *Nhe*I restriction site in this synthesized fragment), purified from low molecular weight restriction products by electrophoresis, isolated from the gel, and ligated into pETL7 plasmid cut at the same sites, thus preparing a mix of plasmids containing mutant luciferase gene. *Escherichia coli* strain XL1Blue was transformed with the plasmid, and cells were plated on Petri dishes with LB medium containing 100 μg/ml ampicillin. The presence of the desired amino acid substitutions was verified by sequencing.

Preparation and purification of luciferase. *Escherichia coli* BL21(DE3)CodonPlus cells (Stratagene, USA) were transformed by the respective mutant plasmids.

Luciferase was prepared according to the lactose autoinduction protocol [14]. *Escherichia coli* BL21(DE3)CodonPlus cells containing pETL7 plasmid carrying the initial luciferase gene or a gene encoding its mutant form were plated on a dish with LB medium containing 100 μg/ml ampicillin and 1.5% bacto-agar and incubated overnight at 37°C. Three milliliters of LB with 200 μg/ml ampicillin and 1% glucose were inoculated with several colonies, cells were grown for 5-6 h on a shaker at 37°C, 180 rpm, until the cell suspension became turbid (A_{600} = 0.4-1.0). The cell culture was then diluted to A_{600} ~ 0.0024 with 200 ml of ZYP-5052 [14] containing 100 μg/ml ampicillin, and the flask was incubated on a shaker for 2 h at 37°C, 180 rpm, until the suspension was slightly turbid (A_{600} = 0.2-0.5). Then cells were grown for 15-18 h at 23°C to reach A_{600} = 5-9. Cells were harvested by centrifugation (5500*g*, 10 min, 4°C). The pellet was resuspended in 18-20 ml of 20 mM Na-phosphate buffer containing 0.5 M NaCl, pH 7.5 (HB buffer), with 20 mM imidazole and 0.5% Triton X-100 added, sonicated (6 cycles for 30 sec, 1 min intervals), and pelleted (39,000*g*, 30 min, 4°C). Supernatant (~20 ml) was loaded at 4°C on a 1 ml Ni-IDA column (Amersham, Sweden) and washed with 20-40 ml HB buffer containing 20 mM imidazole. The enzyme was eluted with HB buffer containing 300 mM imidazole. The resulting luciferase solution was supplemented with EDTA (0.5 M, pH 8.0) to 2 mM and DTT (1 M in 10 mM Na-acetate buffer, pH 5.2) to 1 mM. Fractions were stored at 4°C. Luciferase concentration was determined spectrophotometrically by absorbance at 280 nm using an extinction coefficient $A^{0.1\%}_{1\,cm}$ = 0.75 [15].

Luciferase activity was determined on an FB12 luminometer (Zylux, USA) at 22°C using the maximal 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. Then 150 μl of 0.5 mM luciferin in the same buffer was injected, and the intensity of bioluminescence was registered. Activity was expressed in relative light units (RLU) of the luminometer (1 RLU = $2 \cdot 10^5$ quanta/sec). The K_m^{ATP} and $K_m^{LH_2}$ values were determined from the dependences of the maximal bioluminescence intensity on the luciferin concentration (0.5-500 μM) at constant ATP concentration (4 mM) and of the maximal bioluminescence intensity on the ATP concentration (1-5000 μM) at constant luciferin concentration (1 mM), respectively. The K_m^{ATP} and $K_m^{LH_2}$ values were calculated from the Michaelis–Menton equation using nonlinear regression analysis.

Kinetics of thermal inactivation. Twenty microtubes containing 40 μl luciferase solution in 0.05 M Tris-acetate buffer (2 mM EDTA, 10 mM $MgSO_4$, pH 7.8) each were incubated at Gnom thermostat (DNK-Tekhnologiya, Russia) at fixed temperature for up to ~1.5 h. The sample volume was chosen so that the time period required for the sample to achieve the temperature of interest was negligible compared to the period of sample incubation. At given times a microtube was taken out of the thermostat, cooled on ice for 15 min, and then the enzyme activity was determined.

Bioluminescence and fluorescence spectra were obtained using an LS 50B spectrofluorometer (Perkin-Elmer, England) in "bioluminescence" or "fluorescence" mode, respectively, at a slit width of 10 nm. The temperature-controlled (25°C) fluorometric cuvette was filled with 1 ml 0.05 M Tris-acetate buffer (2 mM EDTA, 10 mM $MgSO_4$, pH 7.8), containing 1 mM ATP and 0.15 mM luciferin. Then 100 μl of 10 μM luciferase solution was added and quickly stirred. Bioluminescence spectra were recorded in the range of 480-780 nm after the light intensity became constant (~3-5 min after reaction initiation). The same solution was used for fluorescence spectra registration, but 1 μM luciferase solution was added and the registration range was 300-400 nm at λ_{ex} = 290 nm. Spectra were corrected for PMT sensitivity using the Perkin-Elmer FL WinLab program.

Bioinformatics. Multiple alignments of the luciferase amino acid sequences were performed using the ClustalW algorithm [16] (BioEdit 7.0.4.1) [17]. Homology models of the luciferase 3D-structures were obtained previously [8]. Molecular graphics were created with YASARA (www.yasara.org) and PovRay (www.povray.org). Analysis of the hypothetical hydrogen bond system was conducted using the Protein Interactions Calculator server [18].

RESULTS

Analysis of luciferase amino acid sequence fragments containing cysteine residues. Among the luciferases whose amino acid sequences are included in the databases, firefly luciferases from *Luciola* and *Hotaria* genera and the *Lampyroidea maculata* firefly luciferase may be considered as a separate group with more than 80% amino acid identity (Fig. 1). A second group includes luciferases from fireflies of various genera: *Nyctophila*, *Lampyris*, *Photinus*, *Pyrocoelia*, etc. including one of the most studied, *P. pyralis* luciferase. The amino acid identity of luciferases from the first and second group does not exceed 70%.

Amino acid sequences of the firefly luciferases belonging to these groups vary significantly. One of the most evident distinctions is the number and location of cysteine residues. Two residues, C82 and C260, are absolutely conserved in all luciferases. The C393 residue is conserved in all luciferases except for the *Cratomorphus distinctus* luciferase. The C62, 86, and 284 residues are also absolutely conserved in all luciferases of the first group. The C146 residue is conserved in all luciferases of the first group except for the *Luciola lateralis* and *Luciola cruciata* luciferases, in which alanine and tyrosine, respectively, are located at position 146. The C164 residue is conserved in luciferases of the first group except *Luciola lateralis* luciferase, which contains S164. The C86 residue is located in a highly conserved region of luciferases of the first group, near the C82 residue, which is located not far from the active site of the enzyme. Besides, the C86 residue is located near the surface of the protein; the surface area of its side chain accessible to the solvent is about 11.0 Å^2. The residue C146 is of particular interest due to its surface location. Its side chain is exposed into the external environment with accessible surface area as high as 48.2 Å^2. All *Luciola* luciferases possess high amino acid sequence identity. However, in its amino acid sequences there are several small areas whose composition varies significantly. The C62 and C164 residues are located in those areas. These residues are positioned in two α-helixes and are in close proximity with each other (Fig. 2).

Cysteine residues 62, 86, 146, and 164 were chosen for mutagenesis. In terms of the molecule topology, the most suitable cysteine residue substitutions are serine (hydrophilic amino acid) and valine (hydrophobic amino acid) residues. The side chain sizes of these residues are similar to that of cysteine. We had considered serine as the most suitable substitution for C86 and C146 residues since the side chains of these residues are in contact with aqueous solvent. The C164 residue was also substituted for serine since its microenvironment is weakly hydrophilic. Moreover, our previous results [7] suggest that under certain conditions this group becomes available to the solvent. For the C62 residue two mutants were prepared – C62S and C62V.

Preparation and physicochemical properties of mutant luciferases. The recombinant *L. mingrelica* firefly luciferase obtained using the pETL7 plasmid (GenBank No. HQ007050) [8] served as the initial enzyme (WT). This form contains four additional amino acid residues (MASK) on the N-terminus as compared to the recombinant *L. mingrelica* firefly luciferase (GenBank No. S61961). On its C-terminus the AKM sequence is replaced by the SGPVEHHHHHH sequence. Mutant plasmids corresponding to the mutant luciferases with single substitutions C62S, C62V, C86S, C146S, C164S, double substitutions C62/146S, C62/164S, C86/146S, and C146/164S, and triple substitution C62/146/164S were obtained by site-directed mutagenesis using the pETL7 plasmid. *XL1Blue E. coli* strain was used for the

Organism	C62	C82, C86	C146	C164	C260	C284	C393
First luciferase group							
Luciola mingrelica	FDIT**C**RLAEAM	IAL**C**SEN**C**EEFF	VQKTVT**C**IKKIVI	NFGGHD**C**METFI	LGYFA**C**GYRVVML	TLQDYK**C**TSVILV	RRGEI**C**VKGPS
Luciola cruciata	LEKS**CC**LGKAL	IAL**C**SEN**C**EEFF	VQKTVT**T**IKTIVI	DYRGYQ**C**LDTFI	LGYLI**C**GFRVVML	TLQDYK**C**TSVILV	RRGEV**C**VKGPM
Hotaria parvula	FDIT**C**RLAEAM	IAL**C**SEN**C**EEFF	VQKTVT**C**IKTIVI	NFGGHD**C**METFI	LGYFA**C**GYRVVML	TLQDYK**C**TSVILV	RRGEI**C**VKGPS
Hotaria unmunsana	FDIT**C**RLAEAM	IAL**C**SEN**C**EEFF	VQKTVT**C**IKTIVI	NFGGYD**C**METFI	LGYFA**C**GYRVVML	TMQDYK**C**TSVILV	RRGEI**C**VKGPS
Hotaria tsushimana	FDIT**C**HLAEAM	IAL**C**SEN**C**EEFF	VQKTVT**C**IKTIVI	NFGGYD**C**METFI	LGYFA**C**GYRVVML	TMQDYK**C**TSVILV	RRGEI**C**VKGPS
Luciola italica	FDIT**C**RLAEAM	IAL**C**SEN**C**EEFF	VQKTVT**C**IKTIVI	NFGGYD**C**VETFI	LGYFA**C**GYRIVML	TLQDYK**C**TSVILV	RRGEI**C**VKGPS
Lampyroidea maculata	FDIS**C**RLAEAM	IAL**C**SEN**C**EEFF	VQKTVT**C**IKTIVI	NFGGYD**C**VETFI	LGYFA**C**GYRIVML	TMQDYK**C**TSVILV	RRGEI**C**VKGPS
Luciola lateralis	LEKS**CC**LGEAL	IAL**C**SEN**C**EEFF	VQKTVT**A**IKTIVI	DYRGYQ**S**MDNFI	LGYLT**C**GFRIVML	TLQDYK**C**SSVILV	RRGEV**C**VKGPM
Luciola terminalis	LDVS**C**RLAQAM	IAL**C**SEN**C**EEFF	VQKTVT**C**IKTIVI	DYQGYD**C**LETFI	LGYLI**C**GFRIVML	TLADYK**C**NSAILV	RRGEI**C**VKGPM
Second luciferase group							
Photinus pyralis	FEMS**V**RLAEAM	IVV**C**SEN**S**LQFF	VQKKLP**I**IQKIII	DYQGFQ**S**MYTFV	LGYLI**C**GFRVVLM	SLQDYK**I**QSALLV	QRGEL**C**VRGPM

Fig. 1. Fragments of amino acid sequence alignment of various firefly luciferases (regions containing cysteine residues). The numbering corresponds to that of *Luciola mingrelica* luciferase.

storage of plasmids, while *BL21(DE3)CodonPlus* strain was used for the enzyme expression. The WT luciferase and its mutant forms were purified using metal chelate chromatography. The expression level and the specific activity of WT and its C62S, C62V, C164S, C62/146S, and C146/164S mutant forms coincided within the experimental error (Table 2). Specific activity of the C146S mutant was ~15% higher than that of the WT, while its expression level was unaltered. The introduction of the C86S substitution resulted in a decrease of the enzyme expression level (62% compared to WT) and its specific activity (30% compared to WT). The properties of the firefly luciferase with C86/146S substitutions were similar to those of the C86S mutant. Drastic decrease of the expression level and the enzyme specific activity was observed on the introduction of double mutation C62/164S and triple mutation C62/146/164S.

Bioluminescence and intrinsic fluorescence spectra of the WT luciferase and its mutant forms were identical. The kinetic constants of the WT and mutant luciferases (K_m^{ATP} and $K_m^{LH_2}$) were obtained at pH 7.8. This pH value is optimal for the luciferase enzymatic activity. Single mutations had almost no effect on both substrates K_m values with the exception of the C86S mutant, for which, as well as for the C86/146S mutant, 1.5-fold increase of both parameters was observed (Table 3). The simultaneous substitution of C62S and C164S residues in both double and triple mutants led to 30% increase of K_m^{ATP} but did not affect $K_m^{LH_2}$.

The irreversible inactivation of the WT luciferase and its mutant forms was measured in 0.05 M Tris-acetate buffer (2 mM EDTA, 10 mM $MgSO_4$, pH 7.8) at 37 and 42°C at concentration range of 0.01-1 μM. It was shown that the inactivation of the WT luciferase and its mutant forms can be expressed (at 42°C as well as at 37°C) by monoexponential dependence at all enzyme concentrations assayed. The k_{in} values of the WT luciferase and its mutant forms did not depend on the initial luciferase concentration. Stabilization of the enzyme was only observed for the C146S mutant: the k_{in} value decreased twofold at 37°C and decreased by 30% at 42°C (Table 4). At 37°C the k_{in} values of the C62V, C164S, and C146/164S mutants were similar to the k_{in} of the WT luciferase, but at 42°C the k_{in} values of these mutants were

Table 2. Specific activity and expression level of the WT *Luciola mingrelica* luciferase and its mutant forms with single and multiple cysteine residue substitutions

Enzyme, mutant	Specific activity × 10^{10}, RLU/mg	Expression level, %
WT	5.1 ± 0.4	100
C62S	4.9 ± 0.3	96 ± 5
C62V	4.5 ± 0.4	98 ± 7
C86S	1.5 ± 0.2	62 ± 6
C146S	5.8 ± 0.5	103 ± 9
C164S	5.2 ± 0.4	100 ± 7
C62/146S	4.8 ± 0.5	80 ± 6
C62/164S	3.6 ± 0.3	26 ± 5
C86/146S	1.8 ± 0.2	58 ± 5
C146/164S	4.4 ± 0.5	92 ± 8
C62/146/164S	3.2 ± 0.3	39 ± 5

Table 3. Kinetic parameters of the reaction catalyzed by the initial *Luciola mingrelica* luciferase and its mutant forms

Enzyme	K_m^{ATP}, μM	$K_m^{LH_2}$, μM
WT	310 ± 30	61 ± 5
62S	330 ± 10	75 ± 6
62V	350 ± 20	68 ± 5
86S	500 ± 40	103 ± 7
146S	290 ± 20	49 ± 4
164S	320 ± 30	58 ± 4
C62/146S	310 ± 10	55 ± 6
C62/164S	420 ± 30	72 ± 3
C86/146S	500 ± 40	113 ± 5
C146/164S	350 ± 30	63 ± 5
C62/146/164S	400 ± 30	79 ± 3

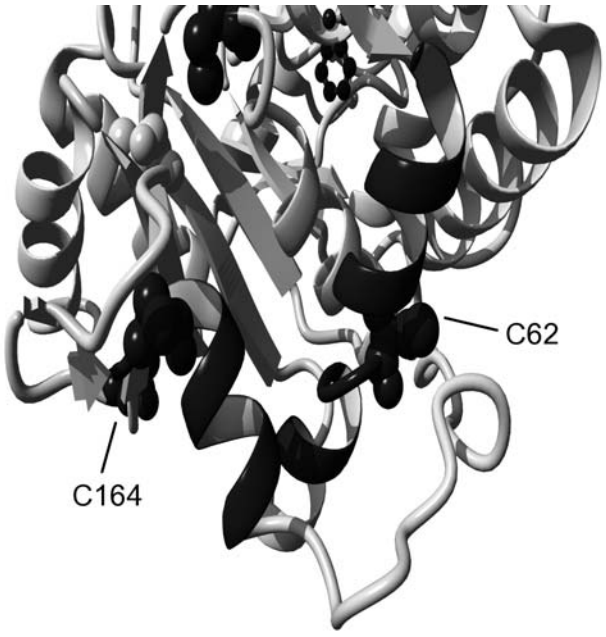

Fig. 2. Fragment of 3D structure of *Luciola mingrelica* firefly luciferase containing C62 and C164 residues.

Table 4. Rate constants of irreversible inactivation of WT luciferase and its mutant forms with single and multiple substitutions of cysteine residues 62, 86, 146, and 164 at 37 and 42°C

Enzyme	k_{in}, min^{-1}	
	37°C	42°C
WT	0.022 ± 0.004	0.074 ± 0.006
C62V	0.024 ± 0.004	0.135 ± 0.004
C62S	0.036 ± 0.004	0.127 ± 0.004
C86S	0.040 ± 0.002	0.160 ± 0.006
C146S	0.011 ± 0.002	0.058 ± 0.003
C164S	0.018 ± 0.003	0.108 ± 0.005
C62/146S	0.042 ± 0.005	0.108 ± 0.005
C62/164S	0.052 ± 0.003	0.153 ± 0.005
C86/146S	0.047 ± 0.004	0.120 ± 0.006
C146/164S	0.023 ± 0.006	0.086 ± 0.005
C62/146/164S	0.055 ± 0.005	0.142 ± 0.006

higher than that of the WT enzyme. All other mutants were less stable than the WT. The C86S substitution caused a significant destabilizing effect on the enzyme: the k_{in} value increased twofold both at 37 and at 42°C. The C62/164S double mutant and C62/146/164S triple mutant were the least stable enzymes among the multiple mutants.

DISCUSSION

Effect of polyhistidine tag on firefly luciferase properties. We previously reported the physicochemical properties of luciferases with single substitutions of the residues C62S, C146S, and C164S obtained on the basis of the *L. mingrelica* luciferase without a His_6-tag (pLR plasmid) [6, 7]. Comparison of the properties of these mutant enzymes and the mutant enzymes containing C-terminus His_6-tag indicates that the His_6-tag has a significant influence on the luciferase properties. Introduction of the His_6-tag into the luciferase structure leads to increase of the K_m^{ATP} and $K_m^{LH_2}$ values. The interaction of the enzyme with the substrates is known to be accompanied by the rotation of a large N-domain and a small C-domain of the luciferase against each other by almost 90° [19]. The N-domain and C-domain approaching is necessary for the participation of the K531 residue from the C-domain in the formation of enzyme–ATP–luciferin active complex. The presence of the flexible His_6-tag on the C-terminus of the molecule might somewhat impede the process of domain rotation, which may result in a slight "deterioration" of the K_m values of both substrates.

Thermal inactivation of the firefly luciferase without His_6-tag is a two-step process, which includes a fast and a slow inactivation stage. The k_{in} values of both stages are dependent upon the enzyme concentration, which is known to be a characteristic feature of oligomeric enzymes. Single mutations C62S, C146S, and C164S result in stabilization of the enzyme at the slow stage of inactivation and in a decrease of dependence of k_{in} upon the enzyme concentration. The thermal inactivation of the His_6-tag containing WT luciferase and its mutants is a one-step process. The k_{in} values of these enzymes do not depend on luciferase concentration and coincide with the k_{in} values of the respective mutants without His_6-tag measured at an increased enzyme concentration (1 μM). The influence of the His_6-tag on the inactivation kinetics of the WT luciferase and its mutants may be referred to the fact that the presence of the His_6-tag considerably modifies the process of luciferase oligomerization.

Effect of the C146S and C164S single substitutions on luciferase thermal stability. The C146S substitution results in twofold stabilization of the enzyme at 37°C and in a 30% increase of the enzyme stability at 42°C. This effect is associated with the surface location of the side chain of this residue, its large solvent accessible area, and lack of interactions with other amino acid residues of the enzyme. The C164S substitution does not alter the enzyme stability at 37°C, but it leads to a destabilization at 42°C, though this destabilization is slighter than that caused by C62V, C62S, and C86S substitutions. This effect is, on one hand, due to the fact, that the C164 residue is located in an area that is distant from the enzyme active site. On the other hand, the temperature rise causes an increase of solvent accessibility, and the replacement of cysteine residue by the hydrophilic serine improves interactions with the solvent.

Difference of properties of C62S and C62V luciferase single mutants. Analysis of the luciferase 3D-model shows that it is hard to estimate unambiguously the character of the C62 residue microenvironment. This residue contacts with both hydrophilic and hydrophobic amino acids. Therefore, two enzymes carrying either a hydrophilic or a hydrophobic side chain in the 62 position were prepared. The specific activity, the expression level, and the kinetic parameters of the C62S and C62V mutants were similar to those of the WT enzyme. The k_{in} values at 42°C were also close, but the C62V mutant turned out to be 1.5-fold more stable than the C62S mutant at 37°C. Therefore, the hydrophobic valine residue is more advantageous at 37°C in terms of the enzyme stability. However, at 42°C the role of the amino acid residue microenvironment in the enzyme stabilization becomes less pronounced and both modifications – serine or valine – results in destabilization of the protein globule.

Role of the C86 residue. The C86S substitution has the most significant influence on the properties of the luciferase. It results in a decrease of the luciferase expres-

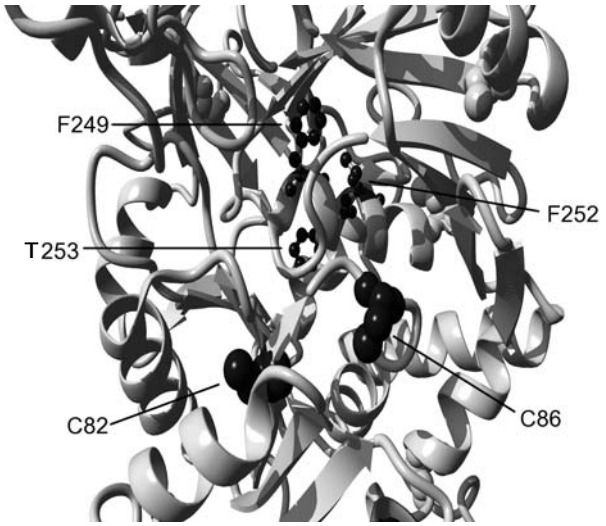

Fig. 3. Fragment of the 3D structure of *Luciola mingrelica* firefly luciferase containing C82 and C86 residues.

sion level and the specific activity, a deterioration of the Michaelis constants of both substrates, and a decrease of the enzyme thermal stability. The C86 residue is located within an unstructured area of the amino acid chain of the enzyme (Fig. 3). The amino acid sequence forms a loop in this area, which is established due to the formation of a hydrogen bond between the C86 residue SH-group and the oxygen atom OE1 belonging to the E88 residue. The SH-group of cysteine residue is known to have a tendency to the formation of nonlinear hydrogen bonds due to fact that the deformation of the valence angle proceeds with relatively small energy cost [20]. The OH-group of serine residues has no such tendency. Thereby it may be possible that the hydrogen bond between S86 and E88 residues cannot be formed in the structure of the C86S mutant. This may lead to an increase of the mobility of the chain fragment containing the abovementioned residues.

It is important to emphasize that the C86 residue is located in an absolutely conserved area of luciferases of the *Luciola* genus, not far from the enzyme active site and at a distance of ~15 Å from the T253, F249, and F252 residues. These residues participate in the process of luciferase substrate binding, and it is known that their mutagenesis leads to a drastic alteration of the enzyme catalytic properties and, in certain cases, to disturbance of the enzyme expression process [21]. On the basis of the experimental data one can conclude that disturbance of the protein structure (the "untwisting" of the helix) in the area of the C86 residue disrupts the native structure of the firefly luciferase active site area and leads to the deterioration of the luciferase activity and stability.

Mutual influence of the C62 and C164 residues. Analysis of the properties of mutants with multiple amino acid substitutions indicates that in most of the cases the effect of such substitutions is additive. For instance, the C86/146S mutant possesses the properties of the luciferase with single C86S substitution, because it is the C86S substitution that affects the enzyme properties the most significantly. The C62/146S and C146/164S mutants also possess the characteristic properties of the respective mutants with single replacements. However, the C62/164S combination leads to a drastic decrease of the enzyme expression level, to the decrease of its specific activity and stability, and to the increase of the K_m^{ATP} compared with the enzymes with C62S and C164S single substitutions. These facts indicate that the effect of these substitutions is non-additive. The analysis of luciferase 3D structure shows that C62 and C164 residues belong to two closely located α-helixes (Fig. 2). Thus, single mutations in the areas in which these residues are located have no significant effect on the enzyme properties. This is probably due to the ability of the enzyme to compensate the effects of these substitutions. Meanwhile, double substitutions affect the mutual disposition of two α-helixes in which these residues are located.

Thus, the role of each cysteine residue in the luciferase molecule is different and is determined by its location relative to the active site, its microenvironment, and the oligomerization state of luciferase.

DNA sequencing was performed at the Inter-Institutional Center "GENOM", Institute of Molecular Biology, Russian Academy of Sciences (http://www.genome-centre.narod.ru) organized with the support of the Russian Foundation for Basic Research (grant 00-04-55000). This work was financially supported by the Russian Foundation for Basic Research (grant 08-04-00624-a and 11-04-00698a).

REFERENCES

1. DeLuca, M. (1976) *Adv. Enzymol.*, **44**, 37-68.
2. Viviani, V. R., and Ohmiya, Y. (2006) in *Photoproteins in Bioanalysis* (Daunert, S., and Deo, S. K., eds.) Wiley-VCH, Weinheim, pp. 49-63.
3. Fraga, H. (2008) *Photochem. Photobiol. Sci.*, **7**, 146-158.
4. Kumita, J. R., Jain, L., Safroneeva, E., and Woolley, G. A. (2000) *Biochem. Biophys. Res. Commun.*, **267**, 394-397.
5. Dement'eva, E. I., Zheleznova, E. E., Kutuzova, G. D., Lundovskikh, I. A., and Ugarova, N. N. (1996) *Biochemistry (Moscow)*, **61**, 115-120.
6. Lomakina, G. Yu., Modestova, Yu. A., and Ugarova, N. N. (2008) *Vestnik Mosk. Univ. Khim.*, **49**, 81-85.
7. Modestova, Y. A., Lomakina, G. Y., and Ugarova, N. N. (2010) *Luminescence*, **25**, 184-185.
8. Koksharov, M. I., and Ugarova, N. N. (2011) *Photochem. Photobiol. Sci.*, **10**, 931-938.
9. Klose, J., Wendt, N., Kubald, S., Krause, E., Fechner, K., Beyermann, M., Bienert, M., Rudolph, R., and Rothemund, S. (2004) *Protein Sci.*, **13**, 2470-2475.

10. Carson, M., Johnson, D. H., McDonald, H., Brouillette, C., and Delucas, L. J. (2007) *Acta Cryst. D*, **63**, 295-300.
11. Amor-Mahjoub, M., Suppini, J. P., Gomez-Vrielyunck, N., and Ladjimi, M. (2006) *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, **844**, 328-334.
12. Freydank, A., Brandt, W., and Drager, B. (2008) *Proteins*, **72**, 173-183.
13. Efremenko, E. N., Lyagin, I. V., Votchitseva, Yu. V., Gudkov, D. A., Peregudov, A. A., Aliev, T. K., and Varfolomeev, S. D. (2008) in *Biotechnology: State of the Art and Prospects for Development* (Zaikov, G. E., ed.) Nova Science Publishers Inc., N. Y., pp. 87-101.
14. Studier, F. W. (2005) *Protein Expression and Purification*, **41**, 207-234.
15. Berezin, I. V., Brovko, L. Yu., and Ugarova, N. N. (1977) *Bioorg. Khim.*, **3**, 1589-1604.
16. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.*, **22**, 4673-4680.
17. Hall, T. A. (1999) *Nucleic Acids Symp. Ser.*, **41**, 95-98.
18. Tina, K. G., Bhadra, R., and Srinivasan, N. (2007) *Nucleic Acids Res.*, **35**, 473-476.
19. Sandalova, T. P., and Ugarova, N. N. (1999) *Biochemistry (Moscow)*, **64**, 962-967.
20. Raso, S. W., Clark, P. L., Haase-Pettingell, C., King, J., and Thomas, G. J., Jr. (2001) *J. Mol. Biol.*, **307**, 899-911.
21. Branchini, B. R., Southworth, T. L., Murtiashaw, M. H., Boije, H., and Fleet, S. E. (2003) *Biochemistry*, **42**, 10429-10436.