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# Enhancement of Thermostability of the *Luciola mingrelica* Firefly Luciferase by Site-Directed Mutagenesis of Nonconservative Cysteine Residues Cys62 and Cys146

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**Abstract**—Mutant forms of the firefly (*Luciola mingrela*) luciferase with point mutations Cys62Ser and Cys146Ser were obtained by site-directed mutagenesis. The mutations did not affect the catalytic activity and fluorescence spectra of the enzyme. The rate constants of the fast ($k_1$) and slow ($k_2$) stages of thermoinactivation of the wild-type and mutant enzymes were determined at 37°C in the absence and presence of 12 mM dithiothreitol (DTT). The thermostability of the mutant forms of luciferase increased several times compared to the wild-type enzyme. In the presence of DTT, $k_2$ of the wild-type enzyme decreased three times whereas neither $k_1$ nor $k_2$ of the mutant forms changed. It was concluded that amino acid residues Cys62 and Cys146 play a major role in luciferase inactivation and that their substitution with Ser stabilizes the enzyme.

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Firefly luciferase belongs to the class of oxidoreductases, subclass of oxygenases. It catalyzes oxidation of *D*-luciferin ($LH_2$) by air oxygen in the presence of ATP and $Mg^{2+}$ [1]. This is one of the most high-specificity biocatalysts and efficient chemical-to-light energy converters. Owing to these properties, this enzyme is widely used in bioanalytical studies [2]. A characteristic feature of luciferases is the presence of free reactive SH-groups of cysteine residues. Luciferases from different sources contain 4–13 free SH-groups; the larger the number of these groups, the smaller the stability of the enzyme [3, 4]. For example, the *P. pyralis* luciferase contains four SH-groups and is more stable than the luciferases of fireflies of the genus *Luciola*, which contain seven or eight SH-groups. Luciferases of Jamaica click-beetles of the family *Elateridae* containing 13 SH-groups are even more instable. Luciferases contain three absolutely conservative Cys residues that are not part of the active site but may considerably affect the activity of these enzymes. For example, mutant *P. pyralis* luciferase in which all the four Cys residues are substituted with Ser retained only 6% of activity, whereas mutants with single substitutions lost 20–60% of activity [5, 6]. However, single substitutions of conserved residues Cys82, Cys260, Cys393 with Ala in *L. mingrelica* luciferase had a negligible effect on the catalytic properties and stability of the enzyme [7]. Apparently, significant differences in the stability of luciferases are determined by the number of nonconservative cysteine residues, especially those in which SH-groups are located in the vicinity or on the surface of the protein globule and are more accessible for oxidation. Titration of SH-groups showed that one cysteine residue is located near the surface of the *P. pyralis* luciferase [8], whereas *L. mingrelica* luciferase contains three surface cysteine residues [9]. Note that addition of dithiothreitol (DTT) slows inactivation of luciferase. This finding indicates that SH-groups of Cys residues are involved in this process. Therefore, it can be expected that substitution of Cys residues with more oxidation-resistant amino acid residues will increase the thermostability of luciferase.

The goal of this study was to determine whether the thermostability of the *L. mingrelica* firefly luciferase can be increased by single substitutions of Cys62 and Cys146 using site-directed mutagenesis.

## MATERIALS AND METHODS

Reagents used in this study were adenosine 5'-triphosphate (ATP), dithiothreitol (DTT) (ICN, United States), *D*-luciferin obtained as described in [10], oligonucleotides (ZAO Sintol, Russia), restriction endonucleases *Nhe* I (Fermentas, Canada) and *Bam*HI (Boehringer Mannheim, Germany), T4 DNA ligase, DNA polymerases Pfu and Pfu Turbo, and a mixture of deoxynucleotide triphosphates (SibEnzyme, Russia). Cells were cultured in LB medium supplemented with ampicillin. Plasmids were isolated from *E. coli* cells and DNA fragments were eluted from agarose gel using Qiagen kits.

**Expression and purification of luciferase mutant forms.** Site-directed mutagenesis was performed by polymerase chain reaction (PCR) using the pLR plasmid carrying the gene for *L. mingrelica* luciferase and

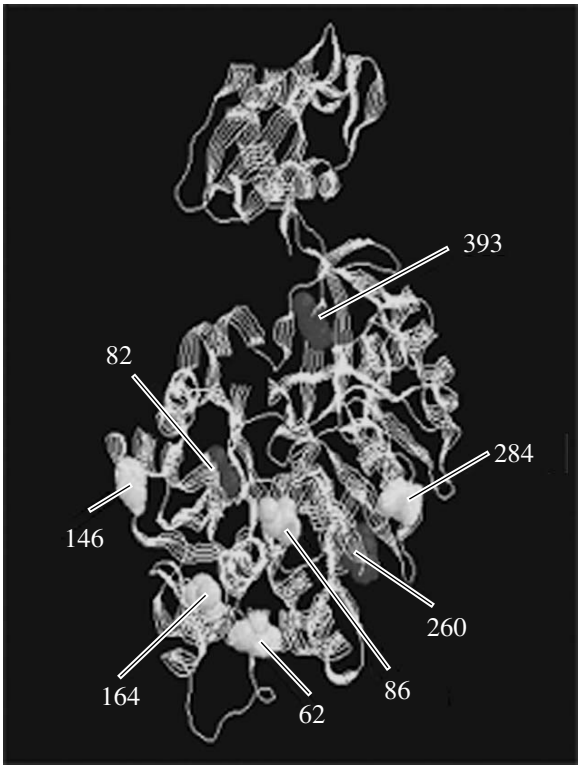


**Fig. 1.** Spatial structure of the *Luciola mingrelica* firefly luciferase.

oligonucleotides 5'-GATATTACATCTCGTTTAGCT-GAGGCCATG-3' and 5'-CCACGATTCTATG-GAAACTTTTATTAAG-3' (to obtain enzymes with point mutations Cys62Ser and Cys146Ser, respectively). Competent cells of the *E. coli* strain LE 392 were transformed with the corresponding plasmid and used for production and purification of preparative amounts of luciferase as described in [7]. High-purity enzyme was stored at –70°C in a working buffer (0.05 M Tris-acetate containing 2 mmol/L EDTA and 10 mmol/L $MgSO_4$ (pH 7.8)). The purity of the enzymes was determined by Laemmli SDS-PAGE in 12% polyacrylamide gel using a Miniprotean II cell (Bio-Rad, United States) with subsequent staining with Coomassie Brilliant Blue R-250. Protein concentration was determined by the optical density measured at 280 nm (0.75 units of optical density corresponded to a luciferase concentration of 1 mg/ml).

**Determination of luciferase activity.** Luciferase activity was determined by the maximum bioluminescence intensity at saturating substrate concentrations (1 mM ATP and 0.3 mM $LH_2$). Bioluminescence intensity was recorded with a Femtomaster FB 12 luminometer (Zylux Corp., United States) [7]. Michaelis constants for luciferin and ATP were determined by varying substrate concentrations in the ranges 0.04–0.4 mM ATP at 1 mM $LH_2$ and 4–40 μM $LH_2$ at 4 mM ATP; the concentration of luciferase was $5 \times 10^{-8}$ M. $K_m$ values were calculated using Origin 6.0 software.

**Study of thermoinactivation of firefly luciferase.** Solutions of the wild-type luciferase and its mutant forms in the working buffer (concentrations $10^{-8}$, $10^{-7}$, and $10^{-6}$ M) were placed into microtubes (30 μl in each) and thermostated at 37°C in the presence and absence of 12 mM DTT. At certain time intervals, the microtubes were taken out of the thermostat and stored on ice until assayed. Luciferase activity was plotted as a function of the time of incubation at 37°C. The obtained kinetic curves were used to calculate the enzyme inactivation rate constants.

**Intrinsic fluorescence spectra of luciferase** were recorded with an LS 50B spectrofluorometer (Perkin Elmer, United Kingdom); the enzyme concentration in the working buffer was $10^{-6}$ M.

## RESULTS AND DISCUSSION

**Expression, purification, and characterization of luciferase mutant forms.** *L. mingrelica* luciferase contains eight Cys residues, three of which (82, 260, and 393) are conservative and five (62, 86, 146, 164, and 284) are nonconservative (Fig. 1). Analysis of the spatial structure of luciferase molecule showed that residues Cys62 and Cys 146 are located on the surface of the protein globule and have a hydrophilic environment. In view of this, for point mutation experiments we have chosen oxidation-resistant serine, whose hydrophilic properties and size are similar to those of cysteine. Mutant plasmids encoding point mutations Cys62Ser and Cys146Ser were obtained by PCR using pLR plasmid carrying the gene for *L. mingrelica* luciferase. Oligonucleotides 27 or 28 bp in length with a substitution of one nucleotide (TGT with TCT) were used. *E. coli* strain LE392 was used for storing plasmids and luciferase expression. The wild-type enzyme and its mutant forms were purified chromatographically. The yield of the active enzyme in all cases was 45–60 mg per liter of nutrient medium. The purity of luciferase preparations, according to electrophoretic data, was 90–95%. The specific activity of the mutant enzyme carrying point mutation Cys62Ser was the same as the activity of the wild-type enzyme, whereas the activity of the mutant carrying point mutation Cys146Ser was 1.5 higher. The Michaelis constants for both substrates of the wild-type and mutant luciferases coincided within the limits of error: $K_{m,\,LH_2} = 20 \pm 3$ μM and $K_{m,\,ATP} = 0.18 \pm 0.04$ mM. Thus, these mutations did not affect the affinity of the enzyme for the substrates. This phenomenon can be explained by the fact that residues Cys62 and Cys146 are remote from the active site of the enzyme by more than 30 Å and do not have a marked effect on its conformation. The intrinsic fluorescence spectra ($\lambda_{max} = 340$ nm) of the wild-type luciferase and its mutant forms also almost did not differ. Therefore, the mutations at residues Cys62 and Cys146 did not alter the microenvironment of Trp419, which is located in the vicinity of the active site of luciferase.

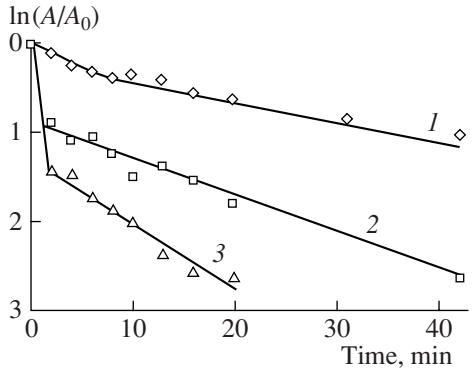


**Fig. 2.** Kinetic curves of thermoinactivation of the wild-type *Luciola mingrelica* luciferase at 37°C in the absence of dithiothreitol at enzyme concentrations (*1*) $10^{-6}$ M, (*2*) $10^{-7}$ M, and (*3*) $10^{-8}$ M. For other reaction conditions, see Table 1.

**Inactivation of *L. mingrelica* luciferase at 37°C.** Thermoinactivation of the wild-type luciferase and its mutant forms was studied at 37°C at different enzyme concentrations ($10^{-6}$–$10^{-8}$ M) in the presence and absence of DTT. The inactivation kinetic curves are described by two exponents corresponding to the fast and slow stages of inactivation (Figs. 2, 3). This pattern of kinetic curves and the inflection point on them on a semilogarithmic scale are characteristic of oligomeric enzymes, a representative of which is firefly luciferase [11–13]. Rate constants of the fast ($k_1$) and slow ($k_2$) stages of inactivation of the wild-type enzyme (Table 1) depend on the enzyme concentration: the higher the concentration, the higher the stability of the enzyme. At high enzyme concentration ($10^{-6}$ M), the inflection point on the curve is less marked. This phenomenon can be explained by the existence of more stable oligomeric forms of luciferase under these conditions. Note that rate constants $k_1$ and $k_2$ of the wild-type and mutant forms of luciferase were similar. When the enzyme concentration is decreased to $10^{-7}$ M and lower, $k_1$ and $k_2$ values increase for both the wild-type and mutant luciferases. The larger the initial concentration of luciferase, the higher the degree of its inactivation at the fast stage. This can be explained by dissociation of the oligomeric enzyme to subunits with a lower enzymatic activity [13]. At low concentrations of luciferase, constants $k_1$ and $k_2$ of mutant forms were four to five times

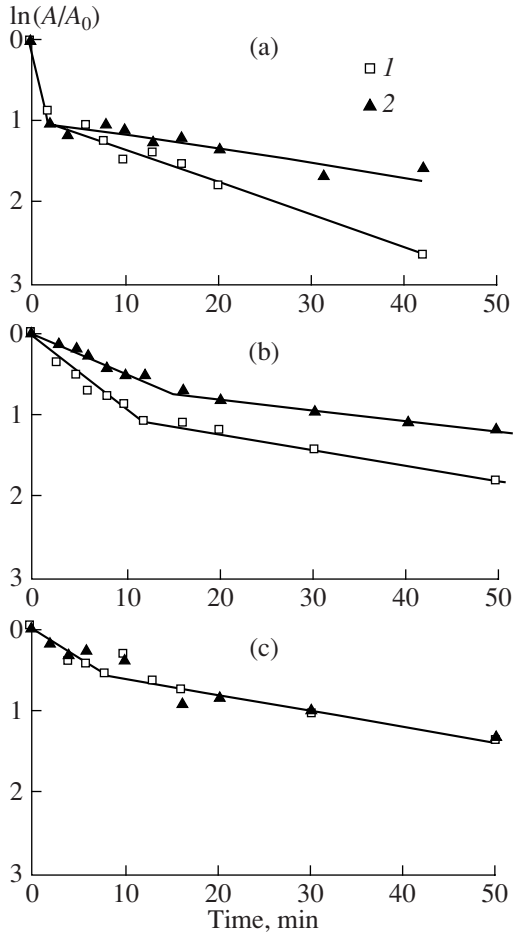


**Fig. 3.** Kinetic curves of thermoinactivation at 37°C of (a) the wild-type *Luciola mingrelica* luciferase and its mutant forms with point mutations (b) Cys62Ser and (c) Cys146Ser in the (*1*) absence and (*2*) presence of 12 mM dithiothreitol. The enzyme concentration was $10^{-7}$ M. For other reaction conditions, see Table 1.

**Table 1.** Rate constants of thermoinactivation of the wild-type *Luciola mingrelica* luciferase and its mutant forms at 37°C in the absence of DTT at different concentrations of the enzyme (reaction conditions: 0.05 M Tris-acetate buffer containing 2 mmol/L EDTA and 10 mmol/L $MgSO_4$ (pH 7.8))

| Enzyme concentration, M | Wild-type enzyme | | Mutant luciferase forms with point mutations | | | |
|---|---|---|---|---|---|---|
| | | | Cys62Ser | | Cys146Ser | |
| | $k_1$, $min^{-1}$ | $k_2$, $min^{-1}$ | $k_1$, $min^{-1}$ | $k_2$, $min^{-1}$ | $k_1$, $min^{-1}$ | $k_2$, $min^{-1}$ |
| $10^{-6}$ | 0.05 ± 0.01 | 0.016 ± 0.005 | 0.06 ± 0.02 | 0.020 ± 0.006 | 0.04 ± 0.01 | 0.012 ± 0.003 |
| $10^{-7}$ | 0.34 ± 0.02 | 0.074 ± 0.003 | 0.10 ± 0.01 | 0.016 ± 0.005 | 0.06 ± 0.01 | 0.016 ± 0.004 |
| $10^{-8}$ | 0.39 ± 0.04 | 0.070 ± 0.009 | 0.16 ± 0.03 | 0.034 ± 0.009 | 0.07 ± 0.02 | 0.015 ± 0.003 |

**Table 2.** Rate constants of thermoinactivation of the wild-type *Luciola mingrelica* luciferase and its mutant forms at 37°C in the presence and absence of 12 mM dithiothreitol (enzyme concentration, $10^{-7}$ M; for other conditions, see Table 1)

| Enzyme | In the absence of DTT | | In the presence of DTT | |
|---|---|---|---|---|
| | $k_1$, $min^{-1}$ | $k_2$, $min^{-1}$ | $k_1$, $min^{-1}$ | $k_2$, $min^{-1}$ |
| Wild-type | 0.34 ± 0.02 | 0.074 ± 0.003 | 0.33 ± 0.03 | 0.023 ± 0.006 |
| Cys62Ser | 0.10 ± 0.01 | 0.016 ± 0.005 | 0.04 ± 0.02 | 0.010 ± 0.005 |
| Cys146Ser | 0.06 ± 0.01 | 0.016 ± 0.004 | 0.05 ± 0.02 | 0.015 ± 0.003 |

lower than those of the wild-type enzyme, and the half-life of the active enzyme ($\tau_{1/2}$) at the slow stage of inactivation increased from 9 min for the wild-type enzyme to 43 min for the mutant forms of luciferase. Thus, mutations Cys62Ser and Cys146Ser significantly increased the stability of luciferase at both stages of inactivation.

It is known that DTT protects SH-groups in enzymes from oxidation and thereby prevents inactivation caused by oxidation of SH-groups. To compare the effect of DTT on the stability of the wild-type and mutant luciferases, we obtained kinetic inactivation curves at an enzyme concentration of $10^{-7}$ M in the presence and absence of 12 mM DTT (Fig. 3). The rate constants calculated for the fast and slow stages of inactivation are summarized in Table 2. In the presence of DTT, $k_1$ did not change, whereas $k_2$ decreased three times (Fig. 3a; Table 2). Therefore, the addition of DTT stabilizes the wild-type luciferase only at the second stage of inactivation. Apparently, in the absence of DTT, $k_2$ is the sum of constants of denaturation and inactivation of the enzyme caused by oxidation of SH-groups in Cys residues, whereas in the presence of DTT $k_2$ is the denaturation constant. Thus, oxidation of SH-groups in Cys residues significantly contributes to luciferase inactivation at the slow stage. In the case of the mutant luciferase carrying point mutation Cys62Ser, the addition of DTT caused a twofold decrease in $k_1$ but almost did not change $k_2$ (Fig. 3b; Table 2). In the case of the mutant luciferase carrying point mutation Cys146Ser, the addition of DTT had no effect on either $k_1$ or $k_2$ (Fig. 3c; Table 2). Therefore, as a result of single substitutions of Cys residues with Ser, both mutant enzymes became insensitive to DTT. Note that, in the presence of DTT, $k_2$ of the wild-type luciferase became similar to $k_2$ of the mutant forms of the enzyme. Thus, the mutant forms carrying point mutations Cys62Ser and Cys146Ser are more stable than the wild-type enzyme both in the presence and absence of DTT. The results obtained in this study indicate that amino acid residues Cys62 and Cys146 are involved in oxidative inactivation of luciferase. Apparently, these residues are required for maintenance of the active conformation of luciferase, and oxidation of SH-groups of these residues leads to its disturbance. Substitution of Cys with Ser resulted in stabilization of the local conformation of the molecule and thereby ensured maintenance of enzymatic activity at 37°C. Elimination of surface Cys residues whose SH-groups are exposed to the solvent abolished the effect of oxidative inactivation of the enzyme at the second, slow stage. In addition, substitution of weakly hydrophobic residues Cys62 and Cys146, located in a hydrophilic environment, with the hydrophilic Ser apparently facilitated the formation of a more compact and stable conformation of luciferase, which is more stable at the first stage of inactivation as well.

Thus, cysteine residues 62 and 146 play a major role in inactivation of luciferase both at the first and second stages of inactivation, and their substitution with the serine residues results in a severalfold stabilization of the enzyme.

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