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Thermostabilization of Firefly Luciferases Using Genetic Engineering

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1. Introduction

Bioluminescence is light emission from living organisms that is based on chemiluminescent reaction catalyzed by a specific kind of enzymes, luciferases. Bioluminescence accompanies the oxidation of an organic substrate called luciferin in the presence of a luciferase enzyme (Wilson & Hastings, 1998). Luciferin and luciferase are generic rather than structural-functional terms that describe substrates and enzymes that interact with each other with the emission of light. Emission of light energy in chemical and biological processes is observed rather often, especially in the reactions with participation of free radicals. However, in most cases the quantum yield (the number of light quanta emitted on the oxidation of one molecule of a substrate) is not higher than tenth or thousandth percent. The distinctive feature and great advantage of bioluminescent systems is high quantum yield (from 1 to 60%), which is achieved by the participation of luciferase in the reaction. Luciferases, on the one hand, act as a matrix, which is covalently or non-covalently bound to emitting chromophore. On the other hand, they play the role of biocatalyst that makes possible the formation of electronically excited product. In most cases luciferases are exceptionally specific to their substrates. The protein structures of bioluminescent enzymes impose strict requirements to the structure of luciferins. This is probably necessary for the maximum concentration of energy of the enzymatic reaction on the chromophore and for the formation of the electronically excited reaction product, which further returns to the ground state with the emission of quantum of visible light.

Luciferases have been isolated from a large number of organisms (insects, fish, bacteria, jellyfish, protozoa, etc) and characterized (Wilson & Hastings, 1998). All of them are oxidoreductases. Molecular air oxygen or hydrogen peroxide acts as an oxidizing agent. The structures of oxidizing substrates, luciferins, are rather different. In most cases, luciferins are heterocyclic compounds. The products of their oxidation have rather low excitation energy, and this is indeed the reason that the energy of enzymatic reaction appears to be enough for the formation of the product in electronically excited state. Therefore, one may conclude that luciferin-luciferase systems are unique, high-performance converters of the energy of biochemical reactions into light, and the interest in the study of structures and functions of luciferases and elucidation of their biological role in nature does not diminishes (Fraga, 2008; Seliger & McElroy, 1960).

The interest for bioluminescent systems is also determined by their great practical importance. For number of luciferases, such important metabolites as flavin mononucleotide (FMN) or adenosine triphosphate (ATP) are necessary participants (co-substrates) of the enzymatic reaction. High quantum yield of bioluminescence in the luciferase-catalyzed reactions simplifies the procedure of light registration. Due to the practically absolute specificity of luciferases to the substrates, luciferases are widely used as highly efficient analytical reagents. The intensity of light emitted in the course of the reaction (bioluminescence intensity) is proportional to the rate of the formation of electronically excited reaction product, and hence is proportional to the concentration of a substrate provided that the substrate concentration is lower than K_m. Since every molecule of the substrate entering the reaction gives from 0.3 to 1 quantum of light, the sensitivity of bioluminescent methods of substrate detection is 10^{-13}–10^{-18} moles of substrates in test solution that is by many orders of magnitude higher than the sensitivity of other detection methods. In addition, the construction of instruments for light measuring (luminometers) is much easier than that of spectrophotometers or spectrofluorimeters, which also simplifies practical application of bioluminescent systems (Ugarova, 2005).

2. Overview of bioluminescent system of fireflies

2.1 Scheme of luciferase-catalyzed reaction

Beetle luciferase [luciferin 4-monooxygenase (ATP hydrolysis); luciferin: oxygen 4-oxidoreductase (decarboxylation, ATP hydrolysis), EC 1.13.12.7] catalyzes firefly luciferin oxidation by air oxygen in the presence of MgATP (Fraga, 2008; Ugarova, 1989). The reaction is accompanied by the emission of visible light with the quantum yield of 40-60% (Niwa et al., 2010). As shown in Fig. 1, at the first step the enzyme binds its substrates luciferin (1) and ATP. After the formation of the triple complex luciferin reacts with ATP to
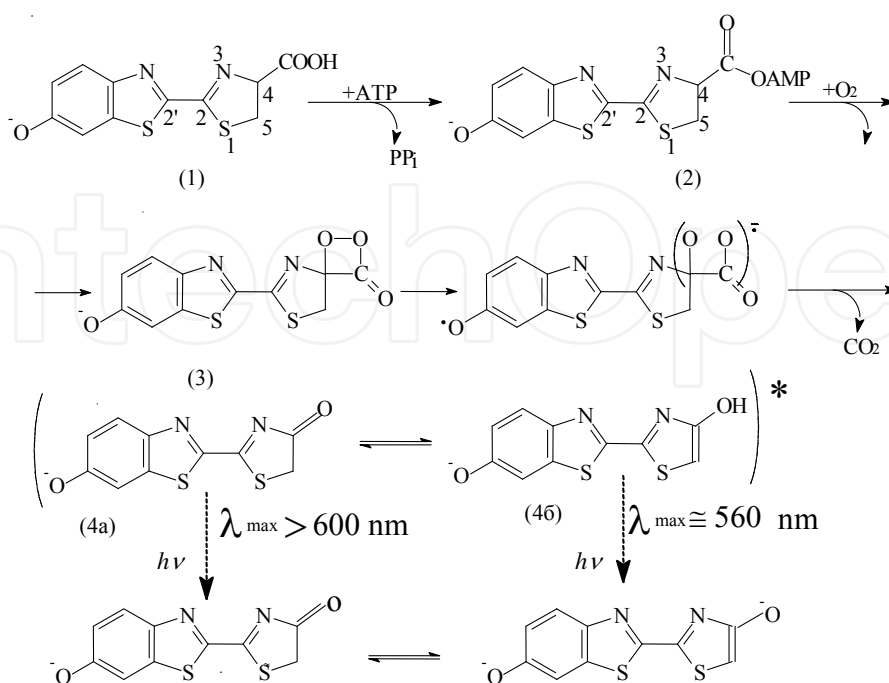

Fig. 1. Scheme of the reaction catalyzed by firefly luciferase (Ugarova et al., 1993)

form mixed anhydride of carboxylic and phosphoric acids, luciferyl adenylate (2) and pyrophosphate (PP_i). The luciferyl adenylate is oxidized with air oxygen to form a cyclic peroxide, dioxytanone (3), through a series of intermediate steps. The dioxytanone molecule has a remarkable feature: one portion of the molecule is a readily oxidizable heterocyclic structure (luciferyl) with low ionizing potential and another portion (peroxide) has a high affinity to electron. Due to the intramolecular electron transfer from the phenolate group to the peroxide, a resonance charge-transfer structure is formed. The break of the O-O bond causes decarboxylation of dioxytanone and formation of biradical (ketone anion-radical and phenolate cation-radical). As a result of intramolecular recombination of the radicals, the reaction product, oxyluciferin (4), is formed in singlet electronically excited state. Depending on the properties of microenvironment, oxyluciferin may exist in the form of ketone (4a), enol (4b), or enolate-anion. The electronically excited oxyluciferin reactivates with the emission of quantum of light with λ_{max} from 536 to 623 nm depending on the firefly species and pH (Viviani, 2002).

2.2 Primary structure of firefly luciferases

Up to the middle of 1980s, the studies were carried out with native firefly luciferases, which were isolated from desiccated firefly lanterns. As a rule, composition of enzyme preparations was not homogeneous due to the presence of different modified forms of enzyme, which, probably, appeared during the functioning of the enzyme. For this reason, even primary amino acid sequence of luciferases remained unknown for a long time. A new phase began in 1985 when cDNA of luciferase for the North American *Photinus pyralis* fireflies was isolated (De Wet et al., 1985). In 1987, the amino acid sequence of this luciferase was determined (De Wet et al., 1987), and in 1989 four luciferases from Jamaican click beetles were cloned (Wood et al., 1989). At the beginning of 1990th, the luciferase from the East-European (north-Caucasian) *Luciola mingrelica* fireflies was cloned and the homogeneous preparation of the recombinant *L. mingrelica* luciferase was obtained (*Devine* et al., 1993). At the present time, primary structures of more than 40 luciferase genes are known that were isolated from different species of fireflies, click-beetles, and railroad worms inhabiting in the USA, Russia, Japan, South-American countries, *etc* (Arnoldi et al., 2010; Oba et al., 2010a; Viviani, 2002). The scheme of the chemical reaction catalyzed and the structure of the emitter are identical for all firefly luciferases. Luciferase molecules consist of one polypeptide chain (542-552 residues), do not contain cofactors and have similar amino acid composition. More than half of the amino acids are non-polar or ambivalent. The number of charged residues is also practically identical for all firefly luciferases, and the main difference between them is in the number of Trp and Cys residues. The amino acid sequence identity for different luciferases usually correlates with phylogenetic relationships of the corresponding fireflies (Li et al, 2006). For luciferases from Luciolini tribe of fireflies (*Luciola* and *Hotaria* genus's) the sequence identity is near 80%, and it is more than 90%for the part of the protein after a 200th residue. The structure of *L. mingrelica* luciferase is very close to the structure of Japanese *Hotaria* (*Luciola*) *parvula* luciferase (98% identity) despite the huge geographic separation of the original firefly species. The *Luciola* luciferases show less identity with luciferases from other firefly subfamilies (67% identity), for example, *P. pyralis* or *L. noctiluca*, and with luciferases from click beetles (43% identity) (Ugarova & Brovko, 2002). At the same time, the identity between the luciferases isolated from Jamaican and Brazil beetles reaches 80% (Oba et al, 2010b; Viviani et al., 1999).

Beetle luciferases belong to the superfamily of enzymes catalyzing formation of acyladenylates from ATP and compounds with a carboxyl group. This superfamily includes the families of non-ribosomal peptide synthetases, acyl-CoA-ligases, *etc* (Gulick, 2009). In 1990s the search of conserved motifs in the amino acid sequences of the proteins within this superfamily has been carried out in our laboratory using computer analysis. In addition to the previously known motif 1 (residues 197-210 in the *L. mingrelica* luciferase), another conserved motif 2 (residues 410-460) has been found. It was proposed that the conserved amino acid residues in motifs 1 and 2 perform key functions in the catalysis and that both motifs belong to a single conserved structural element. It was also proposed that the 197-220 region forms the ATP binding center and the 410-460 region is important for the interaction of the luciferase with its activator coenzyme A (Morozov & Ugarova, 1996). Later structural findings have confirmed these conclusions.

2.3 Spatial structure of firefly luciferase and its complexes with substrates

In 1996 the spatial structure of the luciferase from the American *P. pyralis* fireflies without substrates was obtained using X-Ray analysis (Conti et al., 1996). This opened a new stage in the studies of firefly luciferases. The X-Ray data confirmed the supposition given above about the important role of motifs 1 and 2. Both motifs are loops with undefined electron density indicating high mobility of these fragments, and the motif 2 is partly involved in a polypeptide connecting the two domains of the protein globule. Mutual arrangement of the domains plays an important role in the catalysis as it will be shown below. Unfortunately, one may only assume the possible binding centers for luciferin and ATP. In 1997 the data about the crystal structure of another enzyme belonging to the superfamily of adenylating proteins (adenylase) were published (Conti et al., 1997). In this case the crystals of the complex of the enzyme with its substrates AMP and phenylalanine (analog of the complex of the enzyme with the adenylation product) were analysed. As one may expect, spatial structures of adenylase and luciferase, which both have the same ability for adenylation of substrate carboxyl group with ATP, appeared to be rather similar although the amino acid sequence homology of these enzymes is low. Both enzymes are composed of two domains, large N-domain, which, in turn, may be divided into three sub-domains, and small C-domain. The two domains are connected with very flexible and disordered loop. Each domain and even sub-domain of the adenylase has similar topology with the corresponding domain or sub-domain of the luciferase. The only significant difference is mutual rearrangement of the N- and C-domains. The domains of adenylase are drawn together and rotated by 90º with respect to their orientation in the firefly luciferase (Conti et al., 1997). It was proposed that the observed rotation of domains relative to each other is a consequence of change in the globule conformation upon binding of substrates. In this case the three-dimensional structure of the luciferase-luciferin-ATP complex should be similar to that of the adenylase-phenyl alanine-AMP complex. The computer modeling on the basis of this hypothesis allowed to construct a model of the enzyme-substrate complex for the *P. pyralis* luciferase (Sandalova & Ugarova, 1999). The structures of the luciferase before and after binding of substrates are shown in Fig. 2.

Neither ATP, nor luciferin can bind with the enzyme without the rotation of the domains. The catalytically important residues of the C-domain (Lys529 and Thr527) approach the substrate molecules only after the rotation of the domains. Similar model structure was obtained by Branchini and coworkers (Branchini et al. 1998). All the residues that are in

direct contact with the substrates are absolutely conservative. These models were further confirmed by the independent studies: the residual activity of the luciferase mutant, in which Lys529 was changed to Ala, was less than 0.1% (Branchini et al., 2000). The role of others residues of the luciferase active site was supported by mutagenesis methods (Branchini et al., 1999, 2000, 2001, 2003).
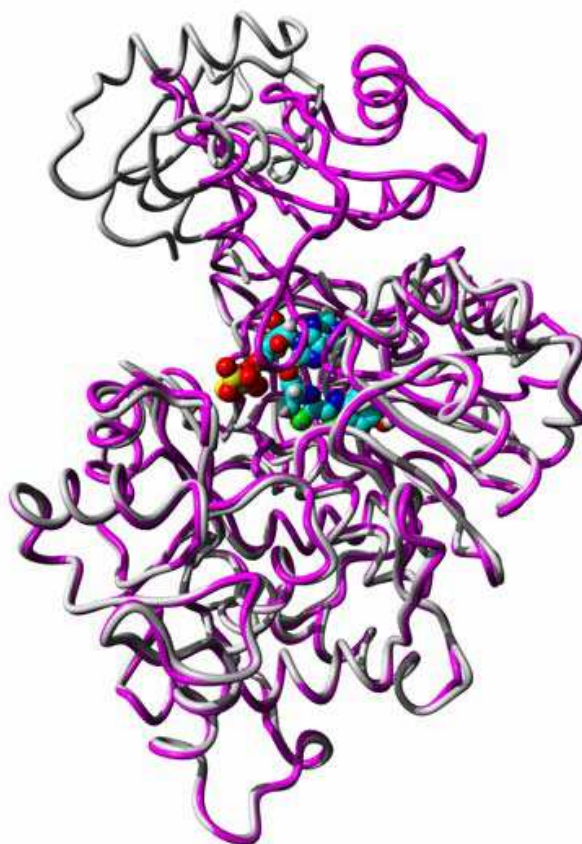

Fig. 2. Superimposition of the spatial structures of firefly luciferase without substrates (grey tubes) and the luciferase-luciferin-ATP complex (magenta tubes). The substrates are shown as CPK models (Sandalova & Ugarova, 1999). Molecular graphics were created with YASARA (www.yasara.org) and PovRay (www.povray.org) software

In 2006 three structures of *Luciola cruciata* luciferase were solved (Nakatsu et al., 2006): in complex with Mg-ATP, in complex with the analog of intermediate product of the adenylation step – DLSA, and in complex with the reaction products – LO and AMP. This allowed to correct the data obtained previously by homology modeling. Firefly luciferase consists of two domains, large N-terminal domain (1-436 amino acid residues) and small C-terminal domain (443-544 amino acid residues), which are connected by the flexible loop (337-442 amino acid residues). By-turn N-terminal domain is composed of two distinct subdomains (Wang et al., 2001): A (1-190) and B (191-436) forming a strong hydrophobic interface (Fig. 3).

When luciferase is in complex with DLSA, the C-domain is rotated by 90° relative to the N-domain compared to the free enzyme. This leads to the transformation from open to closed conformation of luciferase so that both domains are in close contact. The structures obtained allowed to determine the structure of the firefly luciferase active site and the environment of

the substrates at the adenylation step of the reaction. Later findings revealed that yet another rotation of the C-domain by 140° is required for the oxidation step (Gulick, 2009). In this conformation the residue K445 assumes the role of the group K531 (Branchini et al., 2005; Branchini et al., 2011).
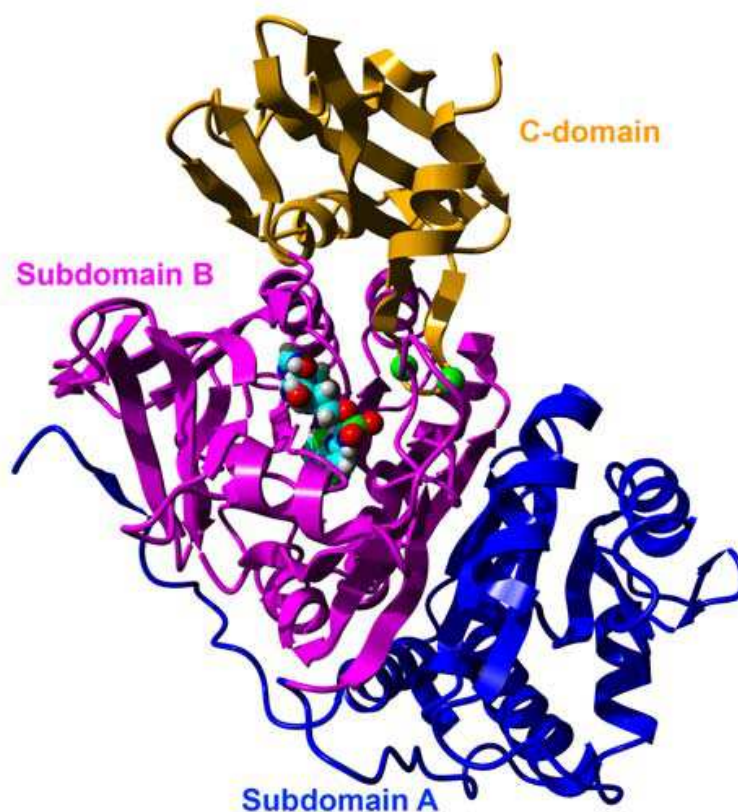

Fig. 3. 3D-structure of *L. cruciata* firefly luciferase in complex with DLSA (Nakatsu et al., 2006). Subdomains A, B and C are depicted in blue, magenta and orange, respectively

3. Stability of firefly luciferases in solution

Firefly luciferases are currently used for microbial contamination testing, as very sensitive reporter genes and in pyrosequencing. They are also promising labels in immunoassays and biosensors for the study of protein-protein interactions (Binkowski et al., 2009; Lundin, 2000; Viviani & Ohmiya, 2006). The stability and activity of enzymes are basic parameters defining the efficiency of their applications. Nevertheless, applications of wild-type firefly luciferases are often limited by the insufficient stability of the enzyme. Firefly luciferases demonstrate low stability in water solutions (Dementieva et al., 1989; White et al., 1996) and can aggregate as well as can be easily adsorbed on the surfaces (Hall & Leach, 1988; Herbst et al., 1998; Suelter & DeLuca, 1983).

The *P. pyralis* luciferase loses up to 99% of activity within 24 hours at 4-8°C as a result of protein adsorption on the container surface when low concentrations of luciferases are used. The addition of bovine serum albumin (0.1 mg/ml) reduces activity loss associated with adsorbtion to 10% even at very low concentrations of luciferase (10 ng/ml). An increase of ionic strength increased the stability of luciferase and an addition of Triton X-100 (0.2 mM)

and glycerol (10-50%) prevented the adsorption of luciferase on the surface (Suelter & DeLuca, 1983). The stabilizing effect of salt addition was also observed for *L. mingrelica* firefly luciferase. In presence of 0.4 M $MgSO_4$ and 0.5 M Na_2SO_4 the thermal stability of *L. mingrelica* luciferase was 2 and 10 times higher respectively compared with a buffer solution without the salt added (Brovko et al., 1982). However, even in the presence of stabilizing salts at 10°C *L. mingrelica* luciferase loses about 90% of activity after 10-15 days storage at the concentration of 0.1 mg/ml. But after the addition of 10% ethylene glycol and the antimicrobial agent (0,02% NaN_3) the luciferase can be stored more than 100 days without loss of activity (Dementieva et al., 1989). The presence of osmolytes in the solution such as glycine betaine and polyols can lead to a significant stabilization of luciferase at the elevated temperatures (Eriksson et al, 2003; Mehrabi et al., 2008; Moroz et al., 2008). Luciferases from the *Luciola* genus readily inactivate at the low ionic strength though *P. pyralis* luciferase is crystallized at the same conditions (Dementieva et al., 1989; Simomura et al., 1977). Native firefly luciferases as well as recombinant enzyme during eukaryotic expression are localized in peroxisomes (De Wet et al., 1987; Gould et al., 1987; Keller et al., 1987) due to the presence of C-terminal signal peptide (AKM, SKL) that is recognized by the cellular peroxisomal transport system (Rainer, 1992).

Firefly luciferase is sensitive to proteolysis, which leads to a short *in vivo* half-life of the enzyme. This effect is especially pronounced at high temperatures when partial enzyme unfolding leads to the higher accessibility of proteolytic sites (Frydman, 1999). Two protease sensitive regions (206-220 and 329-341 residues) were identified in *P. pyralis* luciferase amino acid sequence (Thompson et al., 1997). Half-life of luciferase activity *in vivo* in mammalian cells is only 3-4 hours at 37°C (Leclerc et al., 2000; Thompson et al., 1991). Substrate and competitive inhibitors change the conformation of luciferase, when introduced to prokaryotic and eukaryotic cells, leading to a several-fold decrease of enzyme degradation (Thompson et al., 1991). *P. pyralis* luciferase quickly inactivates in eukaryotic cells at 40-45°C with a half-life of 4-20 min (Forreitor et al., 1997; Souren et al., 1999b). Nevertheless, the stability can be relatively high at moderate temperatures. For example, *L. mingrelica* luciferase produced in frog oocytes had a half-life of about 2 days (Kutuzova et al., 1989).

At the same time even in the solutions with high ionic strength and in the presence of glycerol (10%) and bovine serum albumin (10 mg/ml) *P. pyralis* luciferase loses >90% of activity *in vitro* within 6-20 min at 37-42°C (Nguyen et al., 1989; Thulasiraman & Matts, 1996; Tisi et al., 2002; White et al., 1996). Similar results were obtained for *L. mingrelica* luciferase (Lundovskikh et al., 1998; Ugarova et al., 2000). It was demonstrated that inactivated luciferase is virtually unable to restore activity after cooling and usually aggregates (Minami & Minami, 1999; Schumacher et al., 1996; Souren et al., 1999a, 1999b). Appreciable spontaneous reactivation was only observed for diluted solutions of luciferase that was fully inactivated by guanidine chloride. But reactivation rate was very low and equilibrium was reached after 72 hours. In case of partial denaturation by guanidine chloride the degree of reactivation was low apparently due to aggregation of the enzyme (Herbst et al., 1998). Denatured luciferase can still be effectively reactivated in the presence of different chaperone systems. For instance, in rabbit reticulocyte lysate a refolding of luciferase occurs with a half-period of 8 minutes (Nimmesgern et al., 1993). A detailed mechanism of luciferase inactivation in solution is still unknown. In several works (Frydman et al., 1999; Herbst et al., 1998; Wang et al., 2001) different unfolding intermediates were studied that are formed during chemical denaturation and refolding of *P. pyralis* luciferase. Thermoinactivation

kinetics was studied in detail in case of *L. mingrelica* luciferase (Brovko et al., 1982; Ugarova et al., 2000). It was shown that at elevated temperature *L. mingrelica* luciferase undergoes two-step inactivation: at the first stage a reversible dissociation of luciferase homodimers probably occurs, which is followed by an irreversible inactivation of monomers (Lundovskikh et al., 1998). Luciferases of *Luciola cruciata* and *Luciola lateralis* were isolated and purified in 1992. *L. cruciata* luciferase was found to be similar to *P. pyralis* luciferase in stability, whereas *L. lateralis luciferase* showed significantly higher thermal and pH stability compared with the other two. *L. cruaciata* and *P. pyralis* luciferases were completely inactivated after incubation at 50°C for 30 min. Under the same conditions (10 mM Na-phosphate, 0.2 mM EDTA, 100 mM NaCl, 10% glycerol) the remaining activity of *L. lateralis* luciferase was about 20% (Kajiyama et al., 1992).

Thus, an optimization of the enzyme microenvironment in the solution may increase the stability of luciferases. The more promising approach is thermostabilization of luciferases by genetic engineering methods, which opens wide opportunities of new enzyme forms with enhanced resistance to such environment factors as temperature, organic solvents, and other chemical agents (Ugarova, 2010). It is important to emphasize that comparison of thermostability of mutant and wild-type luciferases must be performed under identical conditions, because the buffer composition and different stabilizing agents (BSA, ammonium sulfate, phosphates, glycerol, etc.) can greatly affect the rate of thermal inactivation.

4. Thermostabilization of firefly luciferases by random and site-directed mutagenesis

In the absence of structural information on a luciferase, random mutagenesis was the most common and efficient approach to obtain mutant proteins with improved stability to the action of temperature, pH, etc. Firefly luciferase is particularly suitable for activity screens owing to the ease with which its bioluminescence activity can be detected (Wood & DeLuca, 1987). Kajiyama and Nakano were the first to use random mutagenesis in an attempt to isolate thermostable mutants of the luciferase from *L. cruciata* fireflies (Kajiyama & Nakano, 1993). The mutagen-treated plasmid was transformed into *E.coli* JM101. After 12 h at 37°C, colonies on LB/ampicillin plates were transferred on nitrocellulose filters, which were put on new agar plates and incubated at 60°C for 30 min. Remaining luciferase activity was determined on filters by photographic method using X-ray films. Three isolated mutants carried the same an amino acid substitution Thr217Ile and were superior to wild-type in thermal and pH stability. Furthermore, its specific activity was increased to 130% of that of the wild-type. In order to examine the effect of amino acid residue substitution at position 217 on the thermostability of *L. cruciata* luciferase, the authors have replaced the residue Thr217 with all possible amino acid residues by site-directed mutagenesis. The thermostability of these mutants correlated well with hydrophobicity of the substituted amino acid residue. Especially, Thr217Leu and Thr217Val luciferases still retained over 75% of activity after 10 min incubation at 50°C. The fact that hydrophilic and large hydrophobic (Trp and Tyr) substitution resulted in an expression yield >1000-fold less than wild-type is also consistent with residue 217 being in a buried, hydrophobic environment (Kajiyama & Nakano, 1993). The amino acid sequences of *L. cruciata* and *L. lateralis* luciferases are 94% identical. To examine the effect of hydrophobic amino acid at position 217 on the thermostability of *L. lateralis luciferase*, three mutants, Ala217Ile, Leu, Val were constructed,

and all of them demonstrated enhanced thermostability (Kajiyama & Nakano, 1994). The *L. lateralis* luciferase mutant Ala217Leu retained over 70% of the initial activity after 60 min incubation at 50°C. Its half-life was about 20 times longer than that of the wild type *L. lateralis* luciferase. Its thermostability was superior to that of the *L. cruciata* luciferase mutant Thr217Leu.

Random mutagenesis was also used to obtain thermostable mutant of *P.pyralis* luciferase. The substitution Glu354Lys increased thermostability of the enzyme 5-fold (White et al., 1996). The substitution of Glu354 with all possible amino acid residues by site-directed mutagenesis showed that the most stable mutants contained Lys or Arg residues. Thus, the substitution of negatively charged residue to positive one in this part of enzyme molecule increased the thermostability of *P.pyralis* luciferase. Thermostable *P.pyralis* luciferase was also obtained by a combination of random and site-directed mutagenesis. The double mutant was constructed that contained the substitutions Glu354Lys and Ala215Leu (similar to Ala217Leu in *L. lateralis* luciferase). In this case the effect of thermostabilization was not as high as for *. lateralis* luciferase. At 37°C the single mutants retained 10-15% of activity after 5 hours, whereas the wild type luciferase was completely inactivated. The double mutant combined the thermostability gains of the single mutants and retained greater than 50% activity for over 5 h. At 42°C the half life of the double mutant was reduced to 20 minutes. At 50°C it was only 4 min (Price et al., 1996). Other point mutations have been identified (largely by random mutagenesis) that significantly increase the thermostability of the *P.pyralis* luciferase: T214A, I232A and F295L. Combining these point mutations with the E354K mutation into the *P.pyralis* gene resulted in mutant luciferase (rLucx4ts) that had an increase in thermostability of about 7°C relative to the wild-type enzyme. Hence, in this case the multiple point mutations led to a cumulative increase in thermostability (Tisi et al., 2002).

After the spatial structure of luciferase was published, it became possible to rationally select specific positions for mutagenesis. For example, in molecule of *P.pyralis* luciferase five bulky hydrophobic solvent-exposed residues, which are all non-conserved and do not participate in secondary-structure formation, were substituted by hydrophilic ones, in particular by charged groups. These substitutions (F16R, L37Q, V183K, I234K and F465R) led to the enzyme with greatly improved pH-tolerance and stability up to 45°C. The mutant showed neither a decrease in specific activity relative to the wild-type luciferase (Law et al., 2006). Introduction of almost all known point mutations (12 residues) enhancing the thermostability of *P. pyralis* luciferase resulted in a highly stable mutant with half-time of inactivation of 15 min at 55°C, whereas wild-type luciferase inactivates within seconds at this conditions (Tisi et al., 2007).

5. Rational protein design approach to produce the stable and active enzyme

Mutations that are efficient in one particular luciferase do not always lead to successful results when applied to other homologous luciferases. For example, the mutation E354R increased the thermal stability of *P. pyralis* luciferase, whereas the corresponding E356R substitution did not affect *H. parvula* luciferase. The substitution A217L in *L. lateralis*, *L. cruciata* and in *P. pyralis* (A215L) firefly luciferases produced fully active and thermostable mutants, but in the case of *H. parvula luciferase* this mutation decreased activity to about 0.1% of the wild type in spite of some increase in thermal stability (Kitayama, et al. 2003). These results are of particular interest for the *L. mingrelica* luciferase because it shares 98%

homology with *H. parvula*. Hence, both enzymes are considered to be almost identical, and the similar effect of this mutation could be expected for *L. mingrelica* luciferase. A rational protein design approach was used to increase thermal stability of *L. mingrelica* luciferase and prevent the detrimental effect of the of the A217L mutation on its activity by combining the mutation A217L with additional substitutions in its vicinity. The three-dimensional structure of the firefly luciferase and the multiple sequence alignment of beetle luciferases were analyzed to identify these additional substitutions (Koksharov & Ugarova, 2011a). Comparison of the A217 environment in *L. mingrelica* luciferase with that of *L. cruciata* and *L. lateralis* luciferases showed only 3 significant differences: G216N, I212L, S398M. Another difference was the change I212L, but it is unlikely to be important because the properties of Leu and Ile are very close. On the other hand, the neighboring residue G216 and the more remote S398 are characteristic for the small subgroup of luciferases very close in homology to *L. mingrelica* luciferase (including *H. parvula* luciferase). We surmised that the elimination of these differences between two groups of luciferases would lead to the A217 environment similar to that of *L. cruciata* and *L. lateralis* luciferases, which could possibly prevent the loss of activity accompanying the substitution A217L. First, we assumed that that changing the neighboring residue G216 would be sufficient to retain the enzyme activity/ Therefore, the double mutant G216N/A217L was constructed. Since this double mutant still showed low activity, we introduced the additional substitution S398M of the less close residue. This led to a stable and active mutant of *L. mingrelica* luciferase (Table 1).

Enzyme	Mutant	Relative specific activity%	Temperature of inactivation	Half-life, min	Reference
Luciola cruciata luciferase	wild-type	100	50 °C	~ 4	Kajiyama& Nakano, 1993
	T217I	130		~ 28	
Luciola lateralis luciferase	wild-type		50 °C	~ 6	Kajiyama & Nakano, 1994
	A217L			~ 125	
Hotaria parvula luciferase	wild-type	100	45°C	~ 18	Kitayama et al., 2003
	A217L	0.074		~ 60	
Luciola mingrelica luciferase	wild-type	100	45°C	13 ± 1	Koksharov & Ugarova, 2011a
	G216N/A217L	10		280 ± 28	
	S398M	106		16.1 ± 1.6	
	G216N/A217L/S398M	60		276 ± 28	

Table 1. Thermal stability of luciferases with substitution of the residue 217 in a 0.05 M Na-phosphate buffer, containing 0.4 M $(NH_4)_2SO_4$, 2 mM EDTA, 0.2 mg/ml BSA, pH 7.8

The residues 216, 217, 398 are located near one of the walls of the luciferin-binding channel (Fig. 4). In the majority of beetle luciferases position 216 is normally occupied with a residue having a side group but in *L. mingrelica* and *H. parvula* luciferases it is occupied with Gly. Glycine is known to be a very destabilizing residue when in internal position of α-helices because of the absence of side group and excessive conformational freedom (Fersht & Serrano, 1993).

Since the G216 is located in the α-helix (Fig. 4) it can be suggested that it makes the surrounding structure less stable and more sensitive to the substitutions of the neighboring

residues. This can explain the unusual decrease in activity in case of the A217L mutation in *Hotaria parvula* luciferase (Kitayama, et al. 2003). The double mutation G216N/A217L resulted in the significant increase of the thermal stability of *L. mingrelica* luciferase, but this mutant retained only 10% of the wild-type activity. The comparison of the environment of residue 217 in the crystal structure of *L. cruciata luciferase* (Nakatsu, et al., 2006) with the homology model of *L. mingrelica* luciferase (Koksharov & Ugarova, 2008) (Fig. 4) shows that internal cavities probably exist in *L. mingrelica* luciferase near the 216 and 398 positions because of the smaller size side groups of the residues in this positions compared to *L. cruciata luciferase*. Additional cavity in the vicinity of S398 could potentially decrease the local conformational stability, make it more flexible and sensitive to the mutations and the changes in the environment. This hypothesis is supported by the higher resistance of the bioluminescence spectrum of the S398M mutant to pH and temperature, which indicates more rigid and stable microenvironment (Ugarova & Brovko, 2002).
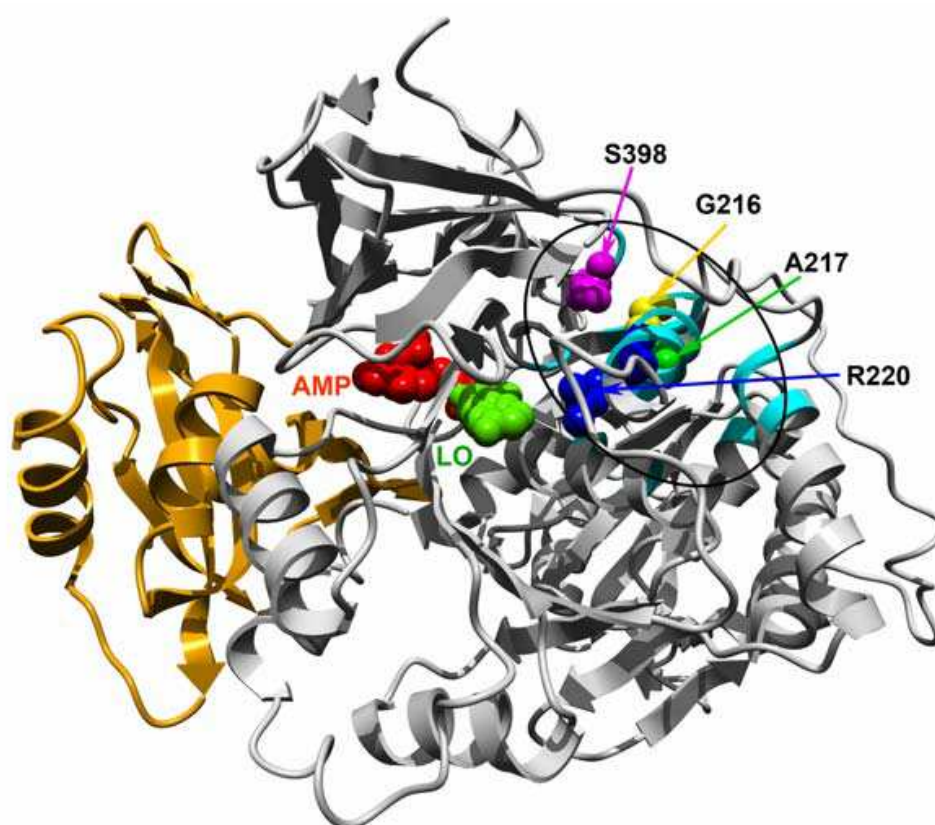

Fig. 4. Structure of *L. mingrelica* luciferase in complex with oxyluciferin (LO) and AMP. The residues G216, A217, R220 and S398 are indicated by arrows. 7 Å microenvironment of A217 is indicated by ellipse (Koksharov & Ugarova, 2011a). The large N-terminal and the smaller C-terminal domains are depicted in grey and orange, respectively

The lowered local conformational stability in the vicinity of G216 and S398 residues can explain why the A217L mutation in *H. parvula* and *L. mingrelica* luciferaess leads to the decline in activity and red shift of λ_{max} that were not observed in the cases of *L. cruciata*, *L. lateralis*, *P. pyralis* luciferases containing Asn or Thr at the position 216 and Met at the position 398. In the former case the enzymes are much more likely to loose the conformation optimal for the activity as a result of residue substitutions. As can be seen the G216, A217,

S398 residues are located in one plane with the neighboring residue R220 (Fig. 5). The residue R220 (the residue R218 in *P.pyralis* luciferase) is highly conservative and necessary for the green emission of firefly luciferases. Its substitutions led to the red bioluminescence, 3-15-fold decrease in activity, extended luminescence decay times and dramatic increase in K_m values (Branchini et al., 2001). The G216N/A217L double substitution in *L. mingrelica* luciferase caused the similar type of effects but of less extent. Thus, in *L. mingrelica* and *H. parvula* luciferases the proper alignment of the R220 residue can be affected by the substitution of A217L and lead to the observed detrimental effects. Placing Asn and Met at positions 216 and 398 respectively (as in the triple mutant G216N/A217L/S398M of *L. mingrelica* luciferase and in native *L. cruciata*, *L. lateralis* luciferases) makes local microenvironment of A217 sufficiently rigid to retain active conformation in the case of the A217L mutation.
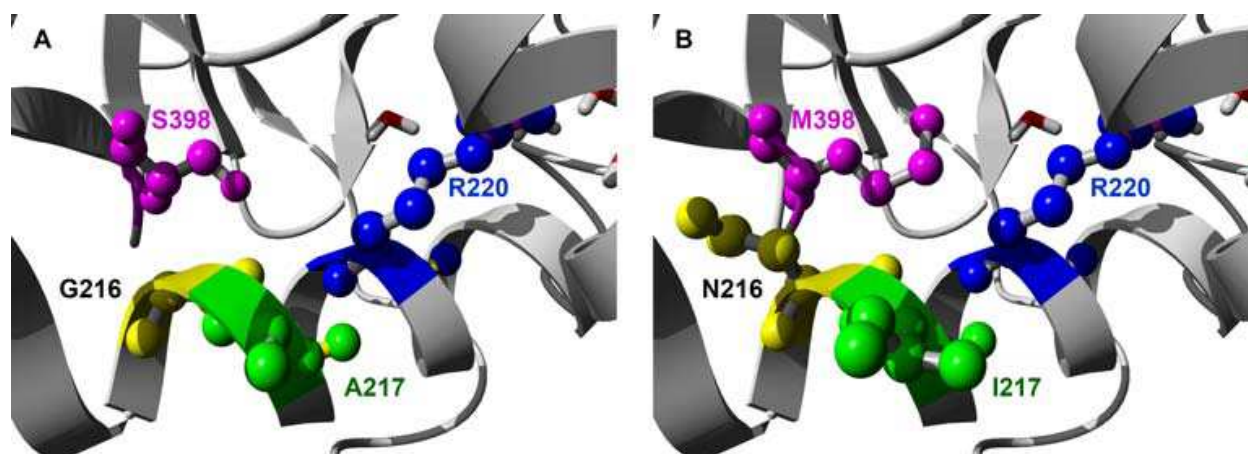

Fig. 5. Residues 216, 217, 220 and 398 in the structures of *L. mingrelica* (A) and *L. cruciata* (B) luciferases (Koksharov & Ugarova, 2011a). Reproduced by permission of The Royal Society of Chemistry (RSC)

In conclusion it can be stated that rational protein design of the residue microenvironment can be an effective strategy when a single mutation in one firefly luciferase does not lead to the desirable effect reported for the mutation of the homologous residue in the another firefly luciferase. The constructed triple mutant G216N/A217L/S398M showed significantly improved thermal stability, high activity and bioluminescence spectrum close to that of the wild-type enzyme. The improved characteristics of this mutant make it a promising tool for *in vitro* and *in vivo* applications.

6. Site-directed mutagenesis of cysteine residues of *Luciola mingrelica* firefly luciferase

The number of Cys residues of luciferases is highly varied (from 4 to 13 residues) depending on the firefly species. Luciferases contain three absolutely conservative SH groups that do not belong to the active site. However their mutagenesis was shown to affect activity and stability of luciferases (Dement'eva et al., 1996; Kumita et al., 2000). For example, the mutant *Photinus pyralis* luciferase in which all the four Cys residues were substituted with Ser, retained only 6.5 % of activity, whereas mutants with single substitutions lost 20-60% of activity (Kumita et al., 2000; Ohmiya & Tsuji, 1997).

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 to alanine were obtained previously (Dement'eva et al., 1996). These substitutions had no effect on bioluminescent and fluorescent spectra of the enzyme and on enzyme activity. The stability of the C393A mutant was 2-fold higher at 5-35°C than that of the wild-type enzyme. The substitutions C82A, C260A did not affect the thermal stability of luciferase. The pLR plasmid, encoding firefly luciferase with the structure identical to that of the native enzyme, was previously used for the preparation of the mutant forms of the enzyme with single substitutions of the non-conserved cysteine residues C62S, C146S (Lomakina et al., 2008) and C164S (Modestova et al., 2010). These substitutions also had no significant effect on the catalytic and spectral properties of the luciferase, but they resulted in an increase of the enzyme thermal stability and in a decrease of the dependence of inactivation rate constant on the enzyme concentration (unlike the wild-type enzyme). Moreover, the DTT influence on luciferase stability was diminished. These effects were most pronounced for the enzyme with the substitution C146S.

The purification of recombinant luciferase obtained using the plasmid pLR is a complicated multistage process. Therefore, the recombinant *L. mingrelica* luciferase with C-terminal His_6-tag was used for mutagenesis of cysteine residues (Modestova et al., 2011). The wild-type enzyme and its mutant forms were expressed in *E. coli BL21(DE3) cells* transformed with the pETL7 plasmid (Koksharov & Ugarova, 2011a). This approach led to the simpler scheme of the luciferase purification and to the increase of the enzyme yield due to the use of the highly efficient pET expression system. The influence of polyhistidine tag on luciferase properties was not previously analyzed in detail according to the literature. A number of publications indicate that while his-tags often don't affect enzyme function, in many cases the biological or physicochemical properties of the histidine tagged proteins are altered compared to their native counterparts (Amor-Mahjoub et al., 2006; Carson et al., 2007; Efremenko et al., 2008; Freydank et al., 2008; Klose et al., 2004; Kuo & Chase, 2011). The goal of this study was to elucidate the role of non-conserved cysteine residues in the *L. mingrelica* firefly luciferase, to study the mutual influence of these residues and the effect of His_6-tag on the activity and thermal stability of luciferase (Modestova et al., 2011).

6.1 Analysis of the fragments of luciferase amino acid sequences containing cysteine residues

Among the firefly luciferases those amino acid sequences are known, firefly luciferases from *Luciola* and *Hotaria* genera, and the *Lampyroidea maculata* firefly luciferase form a separate group with more than 80% amino acid identity (Fig. 6). The second group includes luciferases from fireflies of various genera: *Nyctophila, Lampyris, Photinus, Pyrocoelia,* etc. The sequence identity of luciferases from the first and the 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 amount and location of cysteine residues. The residue C82 is absolutely conserved in all beetle luciferases, and the residue C260 is absolutely conserved in all firefly luciferases. The residue C393 is conserved in all beetle luciferases except the *Cratomorphus distinctus* (Genbank AAV32457) and one (Genbank U31240) of the *P. pennsylvanica* luciferases. The C62, 86, and 284 residues are also absolutely

Origin	C62	C82, C86	C146	C164	C260	C284	C393
First group of luciferases							
Luciola mingrelica	FDITCRLAEAM	IALCSENCEEFF	VQKTVTCIKKIVI	NFGGHDCMETFI	LGYFACGYRVVML	TLQDYKCTSVILV	RRGEICVKGPS
Luciola cruciata	LEKSCCLGKAL	IALCSENCEEFF	VQKTVTTIKTIVI	DYRGYQCLDTFI	LGYLICGFRVVML	TLQDYKCTSVILV	RRGEVCVKGPM
Hotaria parvula	FDITCRLAEAM	IALCSENCEEFF	VQKTVTCIKTIVI	NFGGHDCMETFI	LGYFACGYRVVML	TLQDYKCTSVILV	RRGEICVKGPS
Hotaria unmunsana	FDITCRLAEAM	IALCSENCEEFF	VQKTVTCIKTIVI	NFGGYDCMETFI	LGYFACGYRVVML	TMQDYKCTSVILV	RRGEICVKGPS
Hotaria tsushimana	FDITCHLAEAM	IALCSENCEEFF	VQKTVTCIKTIVI	NFGGYDCMETFI	LGYFACGYRVVML	TMQDYKCTSVILV	RRGEICVKGPS
Luciola italica	FDITCRLAEAM	IALCSENCEEFF	VQKTVTCIKTIVI	NFGGYDCVETFI	LGYFACGYRIVML	TLQDYKCTSVILV	RRGEICVKGPS
Lampyroidea maculata	FDISCRLAEAM	IALCSENCEEFF	VQKTVTCIKTIVI	NFGGYDCVETFI	LGYFACGYRIVML	TMQDYKCTSVILV	RRGEICVKGPS
Luciola lateralis	LEKSCCLGEAL	IALCSENCEEFF	VQKTVTAIKTIVI	DYRGYQSMDNFI	LGYLTCGFRIVML	TLQDYKCSSVILV	RRGEVCVKGPM
Luciola terminalis	LDVSCRLAQAM	IALCSENCEEFF	VQKTVTCIKTIVI	DYQGYDCLETFI	LGYLICGFRIVML	TLADYKCNSAILV	RRGEICVKGPM
Second group of luciferases (illustrated by *Photinus pyralis* luciferase)							
Photinus pyralis	FEMSVRLAEAM	IVVCSENSLQFF	VQKKLPIIQKIII	DYQGFQSMYTFV	LGYLICGFRVVLM	SLQDYKIQSALLV	QRGELCVRGPM

Fig. 6. Fragments of amino acid sequence alignment of various firefly luciferases (the regions containing Cys residues). The numbering corresponds to that of *Luciola mingrelica* luciferase
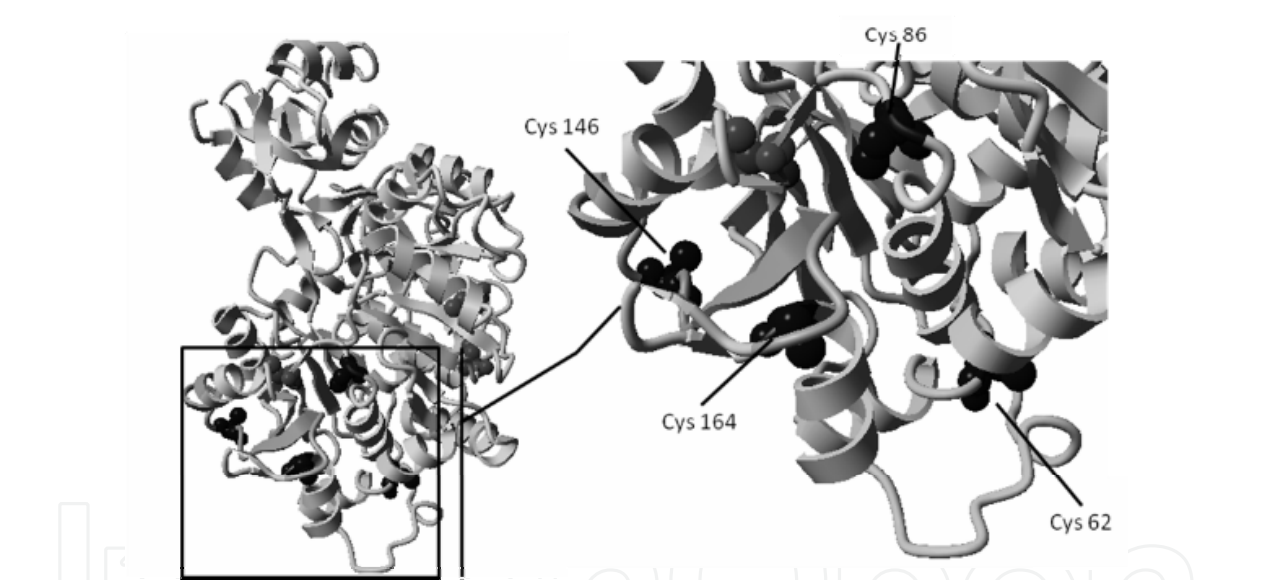

Fig. 7. Fragment of the 3D structure of *Luciola mingrelica* firefly luciferase containing the residues C62 and C164

conserved in all luciferases from the first group. The residue C146 is conserved in all luciferases of the first group, except for the *L. lateralis* and *L. cruciata* luciferases, in which alanine and tyrosine are located at the position 146. The residue C164 is conserved in luciferases of the first group except for the *L. lateralis* luciferase, which contains S146. The C86 residue is located in a highly conserved region of luciferases of the first group, near the C82 residue, which in its turn is located not far from the active site of the enzyme. Besides, the C86 residue is located near the surface of the protein, and the surface area of its side chain, that is accessible to the solvent, is about 11 Å². The residue C146 is of particular interest because of its surface location. Its side chain is exposed to the solvent with the accessible surface area as high as 48 Å². As a whole the *Luciola* luciferases possess high

amino acid sequence identity. However, there are several small areas in their amino acid sequences the composition of which varies significantly. It is in these areas that the residues C62 and C164 are located. These residues are positioned in two α-helixes and are in close proximity with each other (Fig. 7).

The cysteine residues 62, 86, 146, and 164 of *L. mingrelica* luciferase were chosen for the site-specific mutagenesis. In terms of the molecule topology the most suitable substitutions of the Cys are Ser (hydrophilic amino acid) and Val (hydrophobic amino acid). The side chain sizes of these residues are similar to that of Cys. We considered Ser as the most suitable substitution for C86 and C146 residues because the side chains of these residues are in contact with aqueous solution. The residue C164 was also substituted by Ser because its microenvironment is weakly hydrophilic. Moreover, our previously results (Modestova et al., 2010) suggest that in certain conditions this residue becomes available to the solvent. In case of the residue Cys62 two mutants were obtained: C62S and C62V.

6.2 Preparation and physicochemical properties of mutant luciferases

The recombinant *L. mingrelica* firefly luciferase encoded by the plasmid pETL7 (GenBank No. HQ007050) (Koksharov & Ugarova, 2011a) served as the parent enzyme (wild-type). This form contains 4 additional amino acid residues (MASK) on N-terminus as compared to the native sequence of *L. mingrelica* firefly luciferase (GeneBank No. S61961). The sequence AKM at its C-terminus is replaced by the sequence SGPVEHHHHHH. A number of mutants were obtained by site-directed mutagenesis of the plasmid pETL7: the mutant luciferases with the single substitutions C62S, C62V, C86S, C146S, C164S, double substitutions C62/146S, C62/164S, C86/146S, and C146/164S; the triple substitution C62/146/164S. The wild-type luciferase and its mutant forms were purified using metal chelate chromatography. The expression level and the specific activity of wild-type and its mutants C62S, C62V, C164S, C62/146S, and C146S/C164S were the same within an experimental error. Specific activity of the mutant C146S was ~15% higher than that of the wild-type, while its expression level was unaltered. Meanwhile, the substitution C86S resulted in the decrease of the enzyme expression level (62% compared to wild-type) and its specific activity (30% compared to wild-type). The properties of the firefly luciferase with the double substitution C86S/146S were similar to those of the mutant C86S. Drastic decrease of the expression level and of the enzyme specific activity was observed at the introduction of the double mutation C62S/C164S and the triple mutation C62S/C146S/C164S. Bioluminescence and intrinsic fluorescence spectra of the wild-type luciferase and its mutant forms were identical. Single mutations had almost no effect on the K_m values for both substrates (K_m^{ATP} and $K_m^{LH_2}$) with the exception of the mutant C86S, for which, as well as for the mutant C86S/C146S, 1.5-fold increase of both parameters was observed. The simultaneous substitution of the residues C62S and C164S in both double and triple mutants led to 30% increase of K_m^{ATP}, but didn't affect K_m^{LH2}.

The irreversible inactivation of the wild-type luciferase and its mutant forms was measured in 0.05 M Tris-acetate buffer (2 мM EDTA, 10 мM $MgSO_4$, pH 7.8) at 37° and 42°C at concentration range of 0.01-1.0 μM. The inactivation of the wild-type luciferase and its mutant forms followed the monoexponential first-order kinetics at all enzyme concentrations assayed. The k_{in} values of the wild-type luciferase and its mutant forms did not depend on the initial luciferase concentration. The enzyme stabilization was only

observed for the mutant C146S: the k_{in} value decreased 2-fold at 37°C and by 30% - at 42°C (Table 2). At 37°C the k_{in} values of the mutants C62V, C164S and C146S/C164S were similar to the k_{in} of the wild-type luciferase, but at 42°C the k_{in} values of these mutants were higher than that of the wild-type enzyme. All other mutants were less stable than the wild-type enzyme. The substitution C86S caused a significant destabilizing effect on the enzyme: the k_{in} value increased twofold both at 37° and 42°C. The double mutant C62S/C164S and the triple mutant C62S/C146S/C164S were the least stable among the mutants obtained.

Enzyme	k_{in}, min^{-1}	
	37°	42°
wild-type	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
C62S/C146S	0,042 ± 0,005	0,108 ± 0,005
C62S/C164S	0,052 ± 0,003	0,153 ± 0,005
C86S/C146S	0,047 ± 0,004	0,120 ± 0,006
C146S/C164S	0,023 ± 0,006	0,086 ± 0,005
C62S/C146S/C164S	0,055 ± 0,005	0,142 ± 0,006

Table 2. Rate constants of irreversible inactivation of wild-type luciferase and its mutant forms with single and multiple substitutions of the 62, 86, 146, 164 cysteine residues at 37 and 42°C

6.3 The effect of polyhistidine tag on the properties of firefly luciferase

 Comparison of the physicochemical properties of luciferases with single substitutions of the residues C62S, C146S and C164S that were obtained for *L. mingrelica* luciferase without His$_6$-tag (Lomakina et al., 2008) with that of the mutant enzymes containing C-terminal His$_6$-tag (Modestova et al., 2011) led to a conclusion that the His$_6$-tag shows significant influence on the luciferase properties. Introduction of the His$_6$-tag into the luciferase structure leads to the increase of the K_m^{ATP} and K_m^{LH2} values. The interaction of the enzyme with the substrates is known to involve the rotation of a big N-domain and a small C-domain of the luciferase against each other at almost 90° (Sandalova & Ugarova, 1999). This movement is necessary for the participation of the residue K531 from 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 protein molecule might somewhat impede the process of domains rotation, that may result in a slight increase of Km values for the 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 stages. The k_{in} values of both stages are dependent on the enzyme concentration, which is known to be a characteristic feature of oligomeric

enzymes. The single mutations C62S, C146S, C164S result in stabilization of the enzyme at the slow stage of inactivation and in a decrease of k_{in} dependence on the enzyme concentration (Lomakina et al., 2008). The thermal inactivation of the His$_6$-tag containing wild-type 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 that were measured at the increased enzyme concentration (1 µM). This influence of the His$_6$-tag on the inactivation kinetics of the wild-type luciferase and its mutants may be due to the fact that the presence of the His$_6$-tag considerably alters the process of luciferase oligomerization.

6.4 Effect of the cysteine substitutions on luciferase structure and thermal stability

The substitution C146S results in a 2-fold 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 the lack of interactions with other amino acid residues of the enzyme. The C164S substitution doesn't alter the enzyme stability at 37°C, but leads to some destabilization at 42°C, though this destabilization is less than that caused by the substitutions C62V, C62S and C86S. This effect is, on the one hand, due to the fact, that the C164 residue is located in an area, which is distant from the enzyme active site. On the other hand, the raise of temperature causes the increase of solvent accessibility and the replacement of cysteine residue by the hydrophilic serine improves interactions with the solvent.

Analysis of the luciferase 3D-model shows that it is hard to unambiguously estimate the properties of the C62 residue microenvironment. This residue contacts with both hydrophilic and hydrophobic amino acids. Therefore, two enzymes were obtained that carry a hydrophilic and a hydrophobic side chain in the position 62. The specific activity, the expression level and the kinetic parameters of the mutants C62S and C62V were similar to those of the wild-type enzyme. The k_{in} values at 42°C were also similar, but the mutant C62V turned out to be 2-fold more stable than the mutant C62S at 37°C. Therefore, the hydrophobic valine residue is more advantageous at 37°C in terms of the enzyme stability. However, at temperature of 42°C the role of the amino acid residue microenvironment in the enzyme stabilization becomes less pronounced and both modifications – serine or valine – result in destabilization of the protein globule.

The substitution C86S shows the most significant influence on the luciferase properties. It results in a decrease of the luciferase expression level and the specific activity, a deterioration of the K_m values for 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. 8). The amino acid sequence forms a loop in this area due to the formation of a hydrogen bond between the SH-group of the residue C86 and the oxygen atom OE1 belonging to the residue E88. The SH-group of cysteine residue is known to have a tendency to form non-linear hydrogen bonds due to fact that the deformation of the valence angle has a relatively small energy cost (Raso et al., 2001). The OH-group of serine residues has no such tendency. Thereby it may be possible that the hydrogen bond between S86 and E88 residues can't be formed in the mutant C86S. This may lead to an increase in mobility of the chain fragment containing the abovementioned residues.
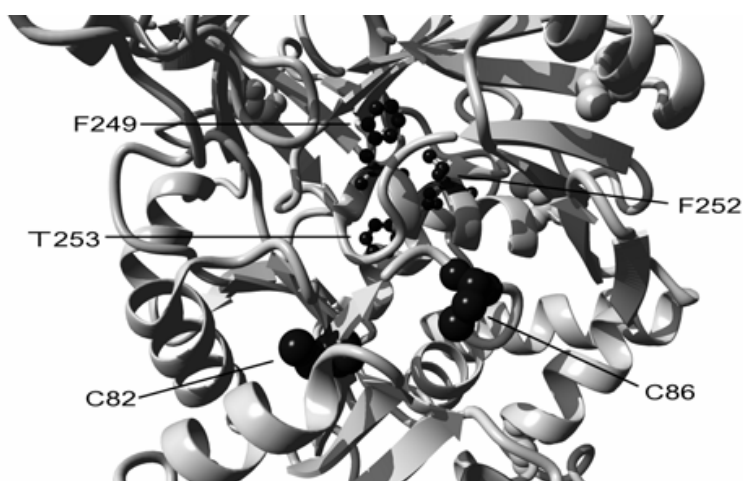
Fig. 8. Fragment of the 3D structure of *Luciola mingrelica* firefly luciferase containing C82 and C86 residues (Modestova et al., 2011)

It is important to underline that the C86 residue is located in an absolutely conserved area of luciferases *Luciola* genus, not far from the enzyme active site and at the distance of ~15 Å from T253, F249, F252 residues. These residues participate in the process of luciferase substrates binding, and it is known that their mutations lead to a drastic alteration of the enzyme catalytic properties and, in certain cases, to the disturbance of the enzyme expression process (Freydank et al., 2008). On the basis of the experimental data one can conclude that disturbance stripping-down of the protein structure (the "untwisting" of the helix) in the area of the localization of the residue C86 disrupts the native structure of the firefly luciferase active site area and leads to the deterioration of the luciferase activity and stability.

Analysis of the properties of the mutants with multiple amino acid substitutions indicates that in most of the cases the effect of such substitutions is additive. For instance, the C86S/C146S mutant possesses the properties of the luciferase with single C86S substitution, because it is the C86S substitution that affects the enzyme properties most significantly. The mutants C62S/C146S and C146S/C164S also possess the characteristic properties of the respective mutants with single replacements. However, the combination C62S/C164S leads to the drastic decrease of the enzyme expression level, to the lowering of its specific activity and stability and to the increase of the K_m^{ATP} in comparison with the enzymes with the single substitutions C62S and C164S. These facts indicate that the effect of these substitutions is nonadditive. The analysis of luciferase 3D structure shows that C62 and C164 residues belong to two closely located α-helixes (Fig. 8). The single mutations of these residues have no significant effect on the enzyme properties, which is probably due to the enzyme ability to compensate the effects of these substitutions. Meanwhile, the double substitutions affect the mutual disposition of two α-helixes, in which these residues are located.

Thus, the role of each cysteine residue in luciferase molecule is different and is determined by its location relative to the active site, its microenvironment and even the oligomerization state of luciferase. For example, in some cases the introduction of Cys residues into internal protein core can increase the luciferase stability after replacement of hydrophilic residue by more hydrophobic Cys. Such examples will be shown below.

7. Increase of *P. pyralis* luciferase thermostability by introduction of disulfide bridges

It was mentioned above that luciferases are peroxisomal enzymes. They do not form structural disulfide bonds despite of containing SH-groups (Ohmiya & Tsuji, 1997). When expressed in *E. coli*, firefly luciferases cannot form any disulfide bonds due to the reducing environment of the cytoplasm. On the other hand, introduction of disulfide bridges was found to be one of the most efficient strategies for increasing protein stability (Eijsink et al., 2004). Recently, disulfide bridges were introduced into *P. pyralis* firefly luciferase (Imani et al., 2010) by site-directed mutagenesis. Two different mutant proteins were made with a single bridge. *P.pyralis* firefly luciferase contains four cysteine residues at the positions 81, 216, 258 and 391. To find the residues capable to form disulfide bridges after their mutation to cysteine, the crystal structure of *P. pyralis* luciferase was uploaded to the NCBS integrated Web Server. The results from server showed that there are 150 pairs that could potentially be selected for disulfide bridge formation. But only two pairs of residues were chosen due to their similar size to the Cys residues: A103 and S121, located distant from active site region of the enzyme, and A296 and A326, situated in the vicinity of the active site region. The ability of mutated sites to form disulfide bridges was analyzed in Swiss-PDB Viewer.

Two mutant luciferases, each containing one S-S bridge, were obtained: A103C/S121C and A296C/A326C. Relative specific activity showed a 7.25-fold increase for the mutant A296C/A326C whereas the mutant A103C/S121C showed only 80% of wild-type specific activity. Both mutants were more stable then the wild-type enzyme. For example, after incubation at 40°C for 5 min the mutants A296C/A326C and A103C/S121C retained ~88% and 22% of activity respectively, whereas the wild-type enzyme lost nearly all of its activity. Using circular dichroism spectropolarimetric and fluorescence spectroscopic analysis, the conformational changes of the enzyme structure were revealed, showing the more fixed structure of aromatic residues, more compactness of tertiary structure, and a remarkable increase in α-helix content.

It can be concluded that disulfide bridge formation in mutant A296C/A326C did not have a destabilizing effect on the enzyme and caused a remarkable change in both secondary and tertiary structure that is reflected in active site structure. These changes endow the enzyme with properties that show an increased resistance to pH and temperature without any stabilizer. On the other hand, the thermal stability of the mutant A103C/S121C arises from the change of tertiary structure. Finally, these results showed that the engineered disulfide bridge not only did not destabilize the enzyme but also in one mutant it improved the specific activity and led to pH-insensitivity of the enzyme (Imani et al., 2010).

8. Thermostabilization of the *Luciola mingrelica* firefly luciferase by *in vivo* directed evolution

Firefly luciferase can be simply screened for its *in vivo* bioluminescence activity (Wood & DeLuca, 1987). This makes a directed evolution approach the most promising for optimization of different luciferase properties including thermostability. This strategy was shown to successful improve of a wide range of properties for different enzymes, for example, thermal stability, enantioselectivity, substrate specificity, and activity in non-natural environments (Jäckel et al., 2008; Turner, 2009). The critical part of a directed

evolution experiment is the availability of a sensitive and efficient screening procedure. Otherwise identifying the desired mutants within large libraries can become very laborious and costly. However, there is only one example known when directed evolution was used for enhancing the thermostability of firefly luciferase. Wood & Hall obtained the exceptionally stable mutant of *Photuris pennsylvanica* luciferase by this approach. This mutant still remains the most stable firefly luciferase to date. In this case a sophisticated automatic robotic system was implemented to screen mutant libraries. It limits the possibility of wide application of this technique. However, that system was able to screen more than 10000 mutants per cycle with a precise measurement of *in vitro* properties of the mutants generated such as activity and K_m. The developed ultra-stable mutant contained 28 substitutions and demonstrated a half-life of about 27 h at 65°C (Wood & Hall, 1999). The more simple, but efficient screening strategy was successfully used here to evolve a thermostable form of *L. mingrelica* luciferase (Koksharov & Ugarova, 2011b).

8.1 Directed evolution of luciferase

Wild-type *L. mingrelica* luciferase displays rather low thermostability with a half-life of 50 minutes at 37°C. So, the consecutive rounds of random mutagenesis and screening were used to considerably improve thermostability of *L. mingrelica* luciferase without compromising its activity. The fact that *E. coli* cells withstand temperatures up to about 55°C (Jiang *et al.*, 2003) and the availability of *in vivo* bioluminescence assay, allowed to identify thermostable mutants by a simple non-lethal *in vivo* screening of *E. coli* colonies that contained mutant luciferases. The incubation of *E. coli* colonies at elevated temperatures resulted in the inactivation of less stable luciferase mutants. Therefore, thermostable mutants displayed higher residual bioluminescence activity and could be efficiently detected by a simple photographic registration of *in vivo* bioluminescence of colonies. *E. coli* cells remained viable after the subjection to elevated temperatures and the subsequent detection of *in vivo* bioluminescence. Therefore, there was no need in using replica plates, which simplified the procedure. Each round of screening could be carried out in a simple and rapid manner (Koksharov & Ugarova, 2010, 2011b).

The plasmid pLR3 (GenBank No. HQ007051) (Koksharov & Ugarova, 2008), which contains *L. mingrelica* luciferase gene, was used in random mutagenesis performed by error-prone PCR. A mutation rate of about 1 amino acid change (2-3 base changes) per the region mutated is reported to be most desirable for an efficient selection of improved mutant (Cirino et al., 2003). It generally gives 30-40% of active clones in the library (Cirino et al., 2003), so this frequency was targeted in our work. Mutagenesis was applied to a 785 bp region of the luciferase gene, which corresponds to amino acid residues 130-390 out of 548 residues of *L. mingrelica* luciferase. This region was chosen because of the convenient restriction sites available (XhoI and BglII) and because most reported mutants, that increase the thermostability of firefly luciferases, are located in this region. The results indicate that the screening of 1000 colonies typically gives a couple of different thermostable mutants. Up to 2000-3000 mutant colonies could be conveniently screened on a single 90 mm Petri dish. The mutant S118C was used as a parent enzyme for directed evolution because it demonstrated slightly higher thermostability compared with the wild-type enzyme (Koksharov & Ugarova, 2008). The most thermostable mutant identified in each cycle of mutagenesis was used as a starting point in the following cycle (Table 3).

Cycle	Parent enzyme	Number of clones screened	Active clones ratio, %	Incubation temperature before screening	Mutant enzyme[*]	Substitutions compared with the parent enzyme
1	S118C	800	53%	37°C	**1T1**	T213S S364C
					1T2 1T3	S364A
2	1T1	900	53%	50°C	**2T1**	K156R A217V
					2T2	E356V
3	2T1	600	65%	50°C	**3T1** 3T2	C146S E356K
					3T3	E356V
4	3T1	1400	65%	55°C	4TS	R211L

[*] For each cycle, the mutant showing the highest stability is shown in bold and underlined. It was used as a parent for the following cycle.

Table 3. Mutants of *Luciola mingrelica* firefly luciferase obtained during four cycles of directed evolution

At the first cycle of mutagenesis the screening of the mutant colonies was performed directly after their growth at 37°C. The wild-type *L. mingrelica* luciferase is insufficiently stable at these conditions, so the *in vivo* bioluminescence of its colonies is rather dim. Three clones were identified during screening that produced distinctly brighter colonies because of the increased thermostability (Table 3). During the second and third cycles of mutagenesis an additional incubation at 50°C for 40 min was required to detect mutants showing higher stability. Three mutants obtained at the third cycle displayed similar brightness after incubation at 50°C but increasing the incubation temperature to 55°C showed that the mutants 3T1, 3T2 are more stable than 3T3. After the fourth round of directed evolution the mutant 4TS was identified, which showed the highest *in vivo* thermostability among the mutants described in this study. It retained noticeable brightness of bioluminescence after incubation of its colonies at 55°C for 40 min while all the other mutants were completely inactivated. Moreover, the mutant 4TS displayed decreased but noticeable *in vivo* bioluminescence when its colonies were heated for 20 min at 60°C. *E. coli* cells completely lost their viability after 2 min at 60°C. Therefore, further selection of mutants with even higher stability will require the of replica plates.

8.2 Expression and purification of mutant and wild-type luciferases

The wild-type *L. mingrelica* luciferase and the mutant 4TS were expressed using the plasmid pETL7, which was described earlier. Average yields of the purified proteins (mg per 1 L of culture) were 160 mg for wild-type and 300 mg for te mutant 4TS. As a result of purification the enzymes were obtained in 20 mM Na-phosphate buffer containing 0.5 M NaCl, pH 7.5 containing 300 mM imidazole, 2 mM EDTA, 1 mM DTT. Generally the luciferases proteins remained fully active for at least 1 month in this buffer. For the long-term storage the

proteins were transferred to 50 mM Tris-acetate buffer (pH 7.3) containing 100 mM Na_2SO_4, 2 mM EDTA and frozen at −80°C. This way they retained full activity for at least 2 years and tolerated several freeze-thaw cycles without inactivation. Despite the fact that the catalytic efficiency of the intermediate mutants was not monitored, the resultant mutant 4TS demonstrated the significant improvement of specific activity as well as K_m for ATP.

8.3 Thermostability

Comparison of 4TS and wild-type *L. mingrelica* luciferase thermal stability at 42°C in Tris-acetate buffer TsB1 (50 mM Tris-acetate buffer containing 20 mM $MgSO_4$, 2 mM EDTA, 0.2 mg/ml BSA, pH 7.8) showed a 65-fold the increase in the half-life of *L. mingrelica* luciferase at 42°C (from 9.1 to 592 min). Thermal inactivation of the wild-type enzyme and 4TS was also studied in Na-phosphate buffer TsB2 (50 mM Na-phosphate buffer containing 410 mM $(NH_4)_2SO_4$, 2 mM EDTA, 0.2 mg/ml BSA, pH 7.8) to compare these results with other literature data (Kajiyama & Nakano, 1994; Kitayama, et al., 2003; White, et al., 1996). At all the temperatures studied the mutant 4TS was significantly more stable than the wild-type. As can be seen from the Arrhenius plot, TsB2 buffer causes significant stabilization of both the wild-type enzyme and 4TS compared with TsB1 buffer (Fig. 9)
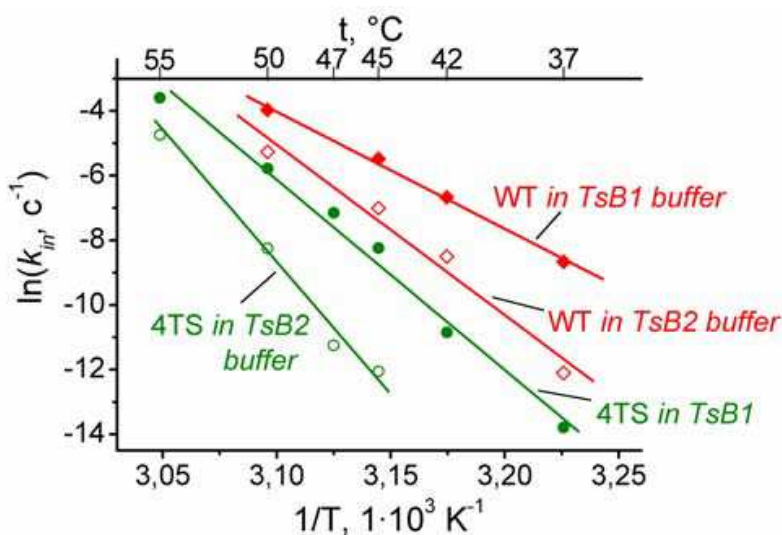

Fig. 9. Arrhenius plot showing the dependence of rates of inactivation on temperature for the wild-type luciferase (diamonds) and the mutant 4TS (circles) in buffer TsB1 (closed symbols) and TsB2 (open symbols) (Koksharov & Ugarova, 2011b). C(enzyme)=13 μg/ml

8.4 Structural analysis

The mutant 4TS contains 7 new substitutions compared with its parent form S118C: T213S, K156R, R211L, A217V, C146S, E356K, and S364C. All the substitutions are non-conservative among firefly luciferases. Judging from the order of appearance of these substitutions in the course of directed evolution (Table 3), literature data and their location in the 3D structure of the enzyme (Fig. 10), four of these substitutions were suggested to be the key mutations that cause the high stability of the mutant 4TS: R211L, A217V, E356K, and S364C. The mutations of the residues A217 (Kajiyama & Nakano, 1993) and E356 (White, et al., 1996) are known to significantly increase the thermostability of firefly luciferases according to the

previous studies. The effect of the residues R211 and S364 on thermostability is identified for the first time. The increase in stability by the substitutions R211L, A217V, S364C, and S364A, can be attributed to the improvement of the internal hydrophobic packing (Fersht & Serrano, 1993). In the case of R211L, S364C, and S364A, the increase of hydrophobicity of the protein core is achieved by the substitution of the non-conservative buried polar residues by the hydrophobic ones. As a result of the substitution A217V the larger side group of Val fills the internal cavity, which is otherwise occupied by a water molecule (Conti et al., 1996). The surface mutation C146S is known to increase the resistance to oxidative inactivation (Lomakina *et al.*, 2008). This mutation can explain the increased storage stability of 4TS in the absence of DTT compared with wild-type. The WT luciferase loses 70% of its activity within two weeks, whereas the mutant 4TS was remained fully active within one month at the same conditions (Koksharov & Ugarova, 2011b). The mutants T213S/S364C and S364A displayed similar *in vivo* properties. There, it the substitution T213S is unlikely to affect thermostability. The substitution of the surface residue 156 from positively charged Lys to similar in properties Arg is also unlikely cause a significant effect on luciferase. The starting mutant S118C showed only small 1.5-fold increase in stability at 42°C. The mutant 4TS and its variant without the mutation S118C showed indistinguishable *in vivo* thermostability at 60°C. Thus, the contribution of S118C seems insignificant. Interestingly, Ser118 is highly
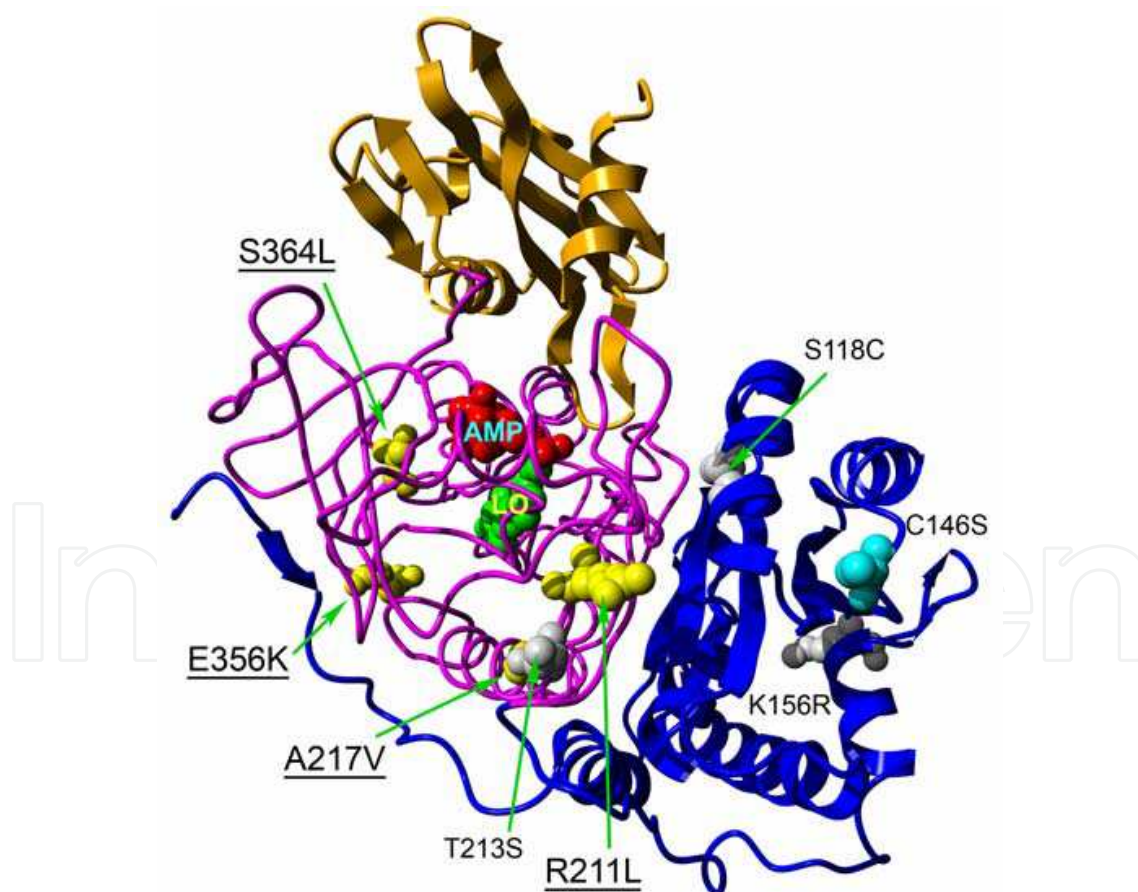

Fig. 10. Homology model of *L. mingrelica* luciferase showing the location of substitutions in the mutant 4TS. Four key thermostabilizing mutations are underlined. LO и AMP – luciferyl and adenylate groups of DLSA (5′-*O*-[N-(dehydroluciferyl)-sulfamoyl] adenosine). Subdomains A, B and C are depicted in blue, magenta and orange, respectively

conservative in firefly luciferases. The only exceptions are the similar substitution S118C in the recently cloned juvenile luciferase from *L. cruciata* (Oba et al, 2010a) and the substitution S118T in the luciferase from *Lampyroidea maculata* (Emamzadeh et al., 2006). However, in luciferases from non-firefly beetles this position is usually occupied by His or Val.

All four key thermostabilizing substitutions (R211L, A217V, E356K, and S364C) are located in the second subdomain of firefly luciferase. According to the results of Frydman and coworkers (Frydman *et al.*, 1999), the fragments of firefly luciferase comprising residues 1-190 and 422-544 possess high intrinsic stability. These fragments mainly correspond to the subdomains A and C of firefly luciferase (Fig. 10). That study demonstrated that the middle subdomain B (192-435) was significantly less stable and that it was the first to unfold under denaturing conditions. Hence, it likely that the stability of the second subdomain is the less stable "bottleneck" that determines the stability of the firefly luciferase protein. Therefore, most of the thermostabilizing mutations would tend to be located in the second subdomain or at the interface of this subdomain and the remaining parts of the protein. It is noteworthy that almost all thermostable mutants reported in the literature are located in this part of the luciferase structure, which is consistent with this hypothesis.

8.5 Conclusion

We have demonstrated that the *in vivo* directed evolution strategy is a simple and efficient method to increase thermal stability of firefly luciferase, which allows to obtain highly thermostable mutants without sacrificing catalytic efficiency. The final mutant obtained here even displayed superior catalytic properties such as higher specific activity, lower K_m for ATP and increased temperature optimum. In typical applications, like ATP-related assays or reporter genes, beetle luciferases are used at room temperature or 37°C. The mutant 4TS retains 70% activity after two days of incubation at 37°C. Therefore, its stability is sufficient for most common *in vivo* and *in vitro* applications. The high specific activity, catalytic efficiency, and improved protein yield make the mutant 4TS an efficient tool for ATP determination (Ugarova *et al.*, 2010). The increased temperature optimum this mutant can be an advantage when used for *in vivo* imaging and in high temperature applications. The new positions identified in this study can be successfully used for the stabilization of other firefly luciferases, especially from the *Luciola* and *Hotaria* genus's. The non-lethal *in vivo* screening approach described here can be potentially implemented to other beetle or non-beetle luciferases when the development of thermostable forms of the enzyme is desirable.

9. Acknowledgements

10. References

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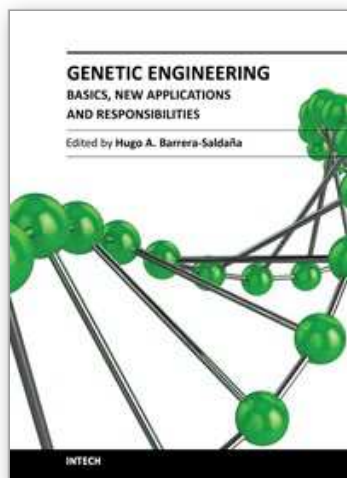
Genetic Engineering - Basics, New Applications and Responsibilities

Edited by Prof. Hugo A. Barrera-Saldaña

Leading scientists from different countries around the world contributed valuable essays on the basic applications and safety, as well as the ethical and moral considerations, of the powerful genetic engineering tools now available for modifying the molecules, pathways, and phenotypes of species of agricultural, industrial and even medical importance. After three decades of perfecting such tools, we now see a refined technology, surprisingly unexpected applications, and matured guidelines to avoid unintentional damage to our and other species, as well as the environment, while trying to contribute to solve the biological, medical and technical challenges of society and industry. Chapters on thermo-stabilization of luciferase, engineering of the phenylpropanoid pathway in a species of high demand for the paper industry, more efficient regeneration of transgenic soybean, viral resistant plants, and a novel approach for rapidly screening properties of newly discovered animal growth hormones, illustrate the state-of-the-art science and technology of genetic engineering, but also serve to raise public awareness of the pros and cons that this young scientific discipline has to offer to mankind.

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