Thermostabilization of firefly luciferase by in vivo directed evolution

Mikhail I.Koksharov¹ and Natalia N.Ugarova
Division of Chemical Enzymology, Department of Chemistry, Lomonosov Moscow State University, Moscow 119991, Russia
¹To whom correspondence should be addressed.
E-mail: mkoksharov@gmail.com

Received April 2, 2011; revised July 26, 2011; accepted August 5, 2011

Edited by David Ollis

Firefly luciferase is widely used in a number of areas of biotechnology and molecular biology. However, rapid inactivation of wild-type (WT) luciferases at elevated temperatures often hampers their application. A simple non-lethal in vivo screening scheme was used to identify thermostable mutants of luciferase in Escherichia coli colonies. This scheme allowed carrying out each cycle of mutagenesis in a rapid and efficient manner. Four rounds of directed evolution were conducted on a part of the gene coding for amino acid residues 130–390 of Luciola mingrelica luciferase. The resultant mutant designated 4TS had a 1.9-fold increase in specific activity, 5.7-fold reduction of half-life of 8.88888C, which is 65-fold higher compared with the WT luciferase. Moreover, the mutant 4TS showed a 1.9-fold increase in specific activity, 5.7-fold reduction of Kₐ for ATP and a higher-temperature optimum compared with the WT enzyme. 4TS contains eight mutations, four of which are suggested to be mainly responsible for the enhancement of thermostability: R211L, A217V, R234Y, and S364C. Thus, directed evolution with non-lethal colony screening for in vivo bioluminescence activity proved to be an effective and efficient approach for increasing thermal stability of luciferase while retaining high catalytic activity.

Keywords: directed evolution/firefly luciferase/luciola mingrelica/random mutagenesis/thermal stability

Introduction

Bioluminescence is widely distributed in nature and can be found in bacteria, dinoflagellates, fungi, insects, coelenterates, fish, mollusks and others. ‘Luciferase’ is a generic term for oxygenases that catalyze the oxidation of a substrate, ‘luciferin’, which is accompanied by the emission of light (Herring, 1987; Hastings and Johnson, 2003). However, different bioluminescence systems are a result of convergent evolution so that the corresponding luciferase proteins are not homologous and the luciferins belong to many unrelated chemical classes. Firefly luciferase (EC 1.13.12.7) catalyzes a two-step oxidation of firefly luciferin (LH₂) in the presence of ATP, Mg²⁺ and molecular oxygen, which results in the emission of a photon and the release of oxyluciferin (LO), AMP, CO₂ and pyrophosphate (Fraga, 2008; Hosseinkhani, 2011). Due to the high quantum yield of its bioluminescence (Niwa et al., 2010), high catalytic efficiency and substrate specificity, firefly luciferase is widely used in ATP assays and as a reporter gene in molecular imaging (Lundin, 2000; Roda et al., 2009; Prescher and Contag, 2010). This enzyme is also a promising tool for molecular sensing of different analytes and protein–protein interactions (Binkowski et al., 2009), for real-time detection of nucleic acid amplification (Gandelman et al., 2010) and a label for immunoassays (Minekawa et al., 2009). However, insufficient stability of wild-type (WT) beetle luciferases at temperatures >30°C often limits their applications demanding the development of thermostable forms of the enzyme (Branchini et al., 2007; Li et al., 2010).

Various approaches have been used to increase the thermostability of firefly luciferase. Improvements in stability at 37°C can be achieved by the addition of stabilizing compounds (Eriksson et al., 2003; Moroz et al., 2008), but the mutagenesis approach appears to be the most successful, especially a random one, since bioluminescence allows simple screening for residual activity. Several specific positions were discovered by random mutagenesis that significantly increased luciferase thermostability (Kajiyama and Nakano, 1993; White et al., 1996; Tisi et al., 2002). In these cases a plate-based screening technique was used to identify thermostable mutants, and bioluminescence of bacterial colonies was detected by the use of an X-ray film (Wood and DeLuca, 1987). However, in the later works an approach of site-directed mutagenesis became the most widespread. The positions identified earlier by random mutagenesis were further explored by site-directed mutagenesis and were used for the construction of multi-point mutants (Tisi et al., 2002; Kitayama et al., 2003; Branchini et al., 2007). The five-point mutant of Photinus pyralis luciferase (Ppl) showed a half-life of 11.5 h at 37°C, a 44-fold improvement over WT (Branchini et al., 2007). In several works the 3D structure of firefly luciferase was used a basis for rational design of thermostable mutants, namely, the mutagenesis of solvent-exposed residues (Law et al., 2006), the introduction of disulfide bonds (Imani et al., 2010) and the comparative analysis of the selected residue microenvironment (Koksharov and Ugarova, 2011).

It should be noted that mutations effective for one firefly luciferase do not always lead to similar results when applied to other homologous luciferases. For example, the A217L substitution in most luciferases resulted in a fully active thermostable enzyme, but in the case of Hotaria parvula luciferase (Hpl) this mutation decreased activity to ~0.1% of the WT enzyme. While the E354R mutation increased thermostability of Ppl, the corresponding E356R substitution did not affect Hpl (Kitayama et al., 2003). Thus, the use of homologous mutations and rational design for the construction of thermostable mutants has some limitations and may require broad study of potential substitutions because many of the designed mutations could be unsuccessful.

Firefly luciferase can be simply screened for its in vivo bioluminescence activity (Wood and DeLuca, 1987), thus...
making a directed evolution approach the most promising. In this strategy multiple consecutive cycles of random mutagenesis and screening are used for an incremental increase of the required property of an enzyme. Directed evolution has been successfully applied to improve or change a wide range of enzyme properties such as expression level, enantioselectivity, substrate specificity, thermal stability and activity in non-natural environments (Kuchner and Arnold, 1997; Eijsink et al., 2005). However, there is only one example when this approach was used to increase the thermostability of firefly luciferase. The most stable firefly luciferase to date is a mutant of Photoris pennsylvanica luciferase obtained by directed evolution. It contains 28 substitutions and shows a half-life of ~27 h at 65°C (Wood and Hall, 1999). In this case a sophisticated automatic-robotic system was used for the screening procedure, thus possibly limiting a wide application of this technique. In this work we report a simple and efficient screening strategy that was successfully used to evolve a thermostable form of Luciola mingrelica luciferase (Lm1).

WT Lm1 displays relatively low thermostability losing ~50% of activity within 50 min at 37°C. We used four consecutive rounds of random mutagenesis and screening to considerably improve thermostability of Lm1 without compromising its activity. Bioluminescent properties of luciferase and the ability of Escherichia coli cells to withstand temperatures up to ~55°C (Jiang et al., 2003) allowed us to identify thermostable mutants by a simple non-lethal in vivo screening of E. coli colonies producing mutant luciferases. Escherichia coli cells remained viable after the screening procedure, so colonies could be picked directly from the plate screened, which eliminates the need for replica plates during primary screening of mutant libraries. Each cycle of mutagenesis was conducted in a rapid and efficient manner by the use of this scheme. The thermostability screening strategy described here is the simplest among those reported in the literature, and can potentially be used to increase the thermostability of any other promising beetle luciferase.

Materials and methods

Materials

Na-ATP, bovine serum albumin, dithiothreitol (DTT), yeast extract (cat. no. Y-0500) were purchased from Sigma-Aldrich (St Louis, USA), α-luciferin was from Lumtek (Moscow, Russia), TaqSE DNA polymerase was from Sibenzyme (Novosibirsk, Russia), Taq DNA polymerase and T4 DNA ligase were from Sileks (Moscow, Russia), oligonucleotide primers were obtained from Sintol (Moscow, Russia), Taq DNA polymerase and T4 DNA ligase buffer, 1 Weiss Unit T4 DNA ligase and 5% w/v PEG-8000 were used as forward and reverse primers, respectively. The PCR reaction mixture (50 μl) contained 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 7 mM MgCl2, 0.2 mM MnCl2, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 20 pmol of each primer, ~2 fmol of pLR3 and 2.5 units of Taq DNA-polymerase.

PCR was performed with an automatic thermal cycler Tercik (DNA-Technology, Russia) under the following conditions: 95°C, 1 min; 25 cycles for 1 min at 94°C, 1.3 min at 53°C, 1 min at 72°C; then 10 min at 72°C. These conditions should lead to an error frequency of two to three substitutions or ~1 amino acid per 800 bp gene region (Miyazaki and Arnold, 1999). The mutagenic PCR product was gel purified by using the QIAEX II kit (Qiagen, Germany) and then digested with XhoI and BglII. This restriction product was gel purified and ligated into pLR3 previously treated with the same restriction enzymes. A typical ligation reaction (10 μl final volume) contained ~20 ng insert, ~50 ng vector, 1×T4 DNA ligase buffer, 1 Weiss Unit T4 DNA ligase and 5% w/v PEG-8000 and was incubated at 16°C for 1 h. Escherichia coli XL1-blue cells were transformed with the resultant mutant plasmids and plated onto Luria–Bertani (LB) agar supplemented with 100 μg/ml ampicillin.

The pLR3 plasmid used in the second to fourth cycles contained the additional mutation Y35N resulting in green bioluminescence of E. coli colonies compared with orange–yellow bioluminescence in the case of WT (Koksharov and Ugarova, 2008). The mutation Y35N was used to identify possible red-shifting mutants during a screening for in vivo bioluminescence. The most stable mutant obtained after the fourth cycle was later subcloned into the plasmid without the mutation Y35N for the subsequent expression and purification.

Thermostability screening

Escherichia coli colonies harboring mutant luciferase genes were grown overnight at 37°C on LB agar plates supplemented with 100 μg/ml ampicillin. The in vivo bioluminescence of the colonies was registered photographically according to the following protocol: plates were filled with a thin layer of 0.5 mM luciferin solution in 0.1 M Na-citrate buffer (pH 5.0), shaken in the dark room and photographed with a Canon PowerShot A530 digital camera (Canon, Malaysia). This step was performed quickly within 3–4 min. Prolonged incubation of the plate under the solution could result in partial washing away of colonies and lead to a cross-contamination. Then the luciferin solution was quickly discarded from the plates and the plates were heated at 50–55°C (depending on the cycle of mutagenesis) for 40 min in Certomat H incubation hood (Sartorius-BBI, Germany). After incubation the colonies were screened for residual activity by photographic registration of the in vivo bioluminescence as written above. The brightest colonies were picked and transferred onto two LB agar plates supplemented with 100 μg/ml ampicillin. Several colonies carrying a parent form of luciferase (in a current cycle of mutagenesis) were also transferred onto these plates as a

Random mutagenesis by error-prone polymerase chain reaction and mutant library construction

The pLR3 plasmid (GenBank No. HQ007051) which encodes Lml gene was constructed earlier (Koksharov and Ugarova, 2008). Random mutagenesis of the 785 base pair (bp) region flanked by XhoI and BglIII restriction sites was performed by error-prone polymerase chain reaction (PCR) (Cirino et al., 2003). Primers 5'-GTATTCAGCTCGAGAAAGGCTTACC-3' and 5'-GCTTGTGTTTCTTAAGATT TCTCTAATTAC-3' were used as forward and reverse primers, respectively. The PCR reaction mixture (50 μl) contained 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 7 mM MgCl2, 0.2 mM MnCl2, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 20 pmol of each primer, ~2 fmol of pLR3 and 2.5 units of Taq DNA-polymerase.

PCR was performed with an automatic thermal cycler Tercik (DNA-Technology, Russia) under the following conditions: 95°C, 1 min; 25 cycles for 1 min at 94°C, 1.3 min at 53°C, 1 min at 72°C; then 10 min at 72°C. These conditions should lead to an error frequency of two to three substitutions or ~1 amino acid per 800 bp gene region (Miyazaki and Arnold, 1999). The mutagenic PCR product was gel purified by using the QIAEX II kit (Qiagen, Germany) and then digested with XhoI and BglII. This restriction product was gel purified and ligated into pLR3 previously treated with the same restriction enzymes. A typical ligation reaction (10 μl final volume) contained ~20 ng insert, ~50 ng vector, 1×T4 DNA ligase buffer, 1 Weiss Unit T4 DNA ligase and 5% w/v PEG-8000 and was incubated at 16°C for 1 h. Escherichia coli XL1-blue cells were transformed with the resultant mutant plasmids and plated onto Luria–Bertani (LB) agar supplemented with 100 μg/ml ampicillin.

The pLR3 plasmid used in the second to fourth cycles contained the additional mutation Y35N resulting in green bioluminescence of E. coli colonies compared with orange–yellow bioluminescence in the case of WT (Koksharov and Ugarova, 2008). The mutation Y35N was used to identify possible red-shifting mutants during a screening for in vivo bioluminescence. The most stable mutant obtained after the fourth cycle was later subcloned into the plasmid without the mutation Y35N for the subsequent expression and purification.

Thermostability screening

Escherichia coli colonies harboring mutant luciferase genes were grown overnight at 37°C on LB agar plates supplemented with 100 μg/ml ampicillin. The in vivo bioluminescence of the colonies was registered photographically according to the following protocol: plates were filled with a thin layer of 0.5 mM luciferin solution in 0.1 M Na-citrate buffer (pH 5.0), shaken in the dark room and photographed with a Canon PowerShot A530 digital camera (Canon, Malaysia). This step was performed quickly within 3–4 min. Prolonged incubation of the plate under the solution could result in partial washing away of colonies and lead to a cross-contamination. Then the luciferin solution was quickly discarded from the plates and the plates were heated at 50–55°C (depending on the cycle of mutagenesis) for 40 min in Certomat H incubation hood (Sartorius-BBI, Germany). After incubation the colonies were screened for residual activity by photographic registration of the in vivo bioluminescence as written above. The brightest colonies were picked and transferred onto two LB agar plates supplemented with 100 μg/ml ampicillin. Several colonies carrying a parent form of luciferase (in a current cycle of mutagenesis) were also transferred onto these plates as a
control. The colonies were grown overnight at 37°C and then stored at room temperature for 4–8 h. Then the colonies were screened for in vivo bioluminescence: one replica plate after the growth to compare the activity of the mutants and another replica plate after heating at 50–55°C for 40 min to identify the most thermostable mutant.

Expression and purification of luciferase

The WT and the mutant 4TS were cloned into the expression vector pETL7 (GenBank No. HQ007050) that was constructed earlier (Koksharov and Ugarova, 2011). The pETL7 plasmid encodes the luciferase protein with several differences as compared with the native enzyme: additional N-terminal sequence MASK- and the C-terminal AKM peptide changed to the SGPVEHHHHH sequence. The WT luciferase and the mutant 4TS were expressed as His6-tagged proteins in E. coli BL21(DE3)CodonPlus cells according to the lactose-based autoinduction method (Studier, 2005).

Escherichia coli cells containing pETL7 plasmid were plated on LB agar containing 100 μg/ml ampicillin and incubated overnight at 37°C. Then 3 ml of LB supplemented with 100 μg/ml ampicillin and 1% glucose were inoculated with one to three colonies. The cultures were grown for 3–4 h on a shaker at 37°C, 180 r.p.m., until the cell suspension became turbid (A600 = 0.3–0.9). One liter flasks with 200 ml of ZYP-5052 medium (Studier, 2005) supplemented with 100 μg/ml ampicillin were inoculated with the cell cultures so that A600 = 0.0024. The 200 ml cultures were grown on a shaker for 2 h at 37°C, 180 r.p.m. or until the suspension became slightly turbid (A600 = 0.2–0.5). Then the cultures were incubated at 23°C for 15–18 h until A600 = 5–9. The cells were harvested by centrifugation at 5500 g and 4°C. The cell pellet was resuspended in 18–20 ml of 20 mM Na-phosphate buffer containing 0.5 M NaCl, pH 7.5 (buffer HB) supplemented with 20 mM imidazole and 0.5% Triton X-100. The cells were lysed by sonication, and the debris was removed by centrifugation at 39 000 g for 30 min. Proteins were further purified using a Ni-affinity column (GE HealthCare, Sweden). The supernatants (~20 ml) were loaded on a 1 ml Ni-IDA column and washed with 20–40 ml HB buffer containing 20 mM imidazole. The enzymes were eluted with HB buffer containing 300 mM imidazole. Chromatography was performed at 4°C. The luciferase solution obtained was further supplemented with EDTA (0.5 M, pH 8.0) to 2 mM and DTT (1 M in 10 mM Na-acetate buffer, pH 5.2) to 1 mM. For the removal of imidazole and the long-term storage, the enzymes were transferred by gel filtration to 50 mM Tris-acetate buffer (pH 7.3) containing 100 mM Na2SO4, 2 mM EDTA (buffer GF). The luciferase solution obtained was further supplemented with 1 mM DTT and stored at −80°C. The protein concentration was determined prior to the addition of DTT by absorbance at 280 nm using an absorption coefficient of A280 = 0.56 for 0.1% solution of Lml, which was calculated based on the amino acid sequence of the protein (Gasteiger et al., 2005).

Bioluminescence spectra

Bioluminescence spectra were obtained using a Perkin-Elmer LS50B luminescence spectrometer operated in the ‘bioluminescence’ mode at a slit width of 10 nm as described previously (Koksharov and Ugarova, 2008). Data were automatically corrected for the spectral response of the R928 photomultiplier tube (PMT) using the FL WinLab software. Generally, the spectra selected for the analysis were recorded when decrease in intensity during the recording interval did not exceed 5%. If the emission was unstable due to the luciferase inactivation at high temperatures, the spectra were corrected for the decrease of intensity during the time of measurement. Spectra were smoothed using Quadratic Golay-Savitzky filter in FL WinLab software.

Enzyme activity and kinetic parameters

Luciferase activity was determined using FB12 luminometer (Zylux, USA). Maximal intensity of the light emitted during the enzymatic reaction at saturating concentrations of substrates (flash height-based activity assay) was used as a measure of the luciferase activity. The cuvette contained 0.35 ml of 1.7 mM ATP in 50 mM Tris-acetate buffer (pH 7.8) containing 2 mM EDTA, 10 mM MgSO4 (buffer AB) and 5 μl of luciferase solution. Assay was initiated by injecting 0.15 ml of 0.5 mM luciferin in the same buffer and the bioluminescence intensity was registered at room temperature (20–25°C). The final concentrations of LH2 and ATP were 0.15 and 1.2 mM, respectively, in a volume of 0.5 ml. An analyzed luciferase solution (1–100 μg/ml) was quickly diluted by the factor of 10^2 or 10^4 just before the measurement so that the bioluminescence intensity values fall within the dynamic range of the luminometer. Activity was expressed in relative light units (RLU/s) of the luminometer. No corrections were applied for the spectral response of the PMT. ATP concentration was calculated using a molar extinction coefficient of 15 400/M/cm at 259 nm in buffer AB. Luciferin concentration was calculated using a molar extinction coefficient of 15 400/M/cm at 385 nm in 0.5 M Na-carbonate buffer, pH 11.5 (Morton et al., 1969).

The values of K_m and V_max for LH2 and ATP were determined from bioluminescence activity assays. The concentration of one substrate was maintained at saturation and the concentration of the other substrate was varied (0.012–1.2 mM ATP and 0.014–0.5 mM LH2). Then the stock solution of 13 μg/ml luciferase was used in the experiment that was further diluted 100-fold during the activity assay. A neutral filter was placed in the cuvette compartment of the luminometer so that bioluminescence intensity values fall within the dynamic range of the luminometer. Kinetic constants were calculated from Michaelis–Menten graph using non-linear regression in Origin 7.5 software (OriginLab, USA).

Kinetics of thermal inactivation

The solution of the purified luciferase (13 μg/ml) was prepared in 50 mM Tris-acetate buffer containing 20 mM MgSO4, 2 mM EDTA, 0.2 mg/ml BSA, pH 7.8 (TsB1 buffer) or in 50 mM Na-phosphate buffer containing 410 mM (NH4)2SO4, 2 mM EDTA and 0.2 mg/ml BSA, pH 7.8 (TsB2 buffer). This solution was then stored on ice at 0°C. In the case of fast inactivation (half-life <30 min) aliquots of 50 μl were placed in eight thin wall 0.5 ml microtubes. Each tube was used for the activity testing separately. In the case of slow inactivation (half-life >30 min) 1.5 ml of luciferase solution was placed in a 1.7 ml microtube. Microtubes were incubated at the temperature studied (37–55°C). At given times a tube with a 50 μl aliquot or 50 μl volume was removed and cooled.
on ice for at least 15 min prior to the activity assay. A neutral filter was placed in the cuvette compartment of the luminometer so that bioluminescence intensity values fall within the dynamic range of the luminometer. The enzyme activity was expressed as a percentage of the initial activity. Half-lives were calculated using the first-order reaction rate constants obtained from semi-logarithmic plots of the percentage of activity versus time.

**Temperature optimum of activity**

The temperature optimum of activity was estimated as follows. The solution of the purified luciferase (5 × 10^{-3} mg/ml) was prepared in 50 mM Tris-acetate buffer (pH 7.8) containing 10 mM MgSO_4, 2 mM EDTA, 0.2 mg/ml BSA and was stored on ice at 0°C. Then 2 ml of 50 mM Tris-acetate buffer (pH 7.8) containing 2 mM EDTA, 10 mM MgSO_4 and 1.7 mM ATP was placed into cuvettes and incubated in a water thermostat for 10 min at the temperature studied. A 10 mM MgSO_4, 2 mM EDTA, 0.2 mg/ml BSA and was prepared in 50 mM Tris-acetate buffer (pH 7.8) containing 2 mM EDTA, 10 mM MgSO_4 and 1.7 mM ATP was placed into cuvettes and incubated in a water thermostat for 10 min at the temperature studied. A cuvette was removed from the thermostat, 10 μl of luciferase solution was immediately added into the cuvette, quickly mixed and placed in the luminometer standing at room temperature. Then 90 μl of 4.5 mM luciferin in the same buffer was immediately injected and the bioluminescence intensity was measured. The integrated intensity during the first 20 s of the reaction was used as a measure of the luciferase activity. The detector of FB12 luminometer is the 9078B PMT (ET Enterprises Ltd, Uxbridge, UK) in photon counting mode with a spectral range of 370–630 nm. It has low and limited sensitivity to red light. Due to the red shift of bioluminescence spectra at elevated temperatures this can lead to the artificial diminishing of relative luciferase activity at increased temperatures. Therefore, the activities at different temperatures were corrected for the PMT sensitivity using the bioluminescence spectra at the corresponding temperatures and the quantum efficiency spectral response curve of the PMT.

**Bioinformatics**

The homology model of the Lml structure was generated previously on its similarity to *Luciola cruciata* luciferase (Lcl) (Koksharov and Ugarova, 2008). The structure of Lcl in complex with DLSA (PDB: 2D1S) (Nakatsu et al., 2006) was used as a template. The primary homology model was generated using What IF server (Rodriguez et al., 1998). A loop region 183–189 of Lml was modeled using ModLoop server (Fiser and Sali, 2003). Molecular graphics were created with YASARA (www.yasara.org) and PovRay (www.povray.org) software.

**Results**

**Screening and selection of thermostable mutants of firefly luciferase**

The key part of directed evolution approach is a sensitive and efficient screening procedure. If a simple screen is available desirable properties can be achieved efficiently thus making this approach superior to the rational design (Kuchner and Arnold, 1997; Eijsink et al., 2005). Otherwise, screening of libraries may become very laborious and costly. In the case of firefly luciferase, incubation of *E. coli* colonies at elevated temperatures led to the inactivation of insufficently stable forms of the enzyme. The subsequent photographic detection of *in vivo* bioluminescence of colonies served as a simple method to identify thermostable mutants that displayed higher residual bioluminescence activity. Since the detection of *in vivo* bioluminescence and elevated temperatures used did not kill *E. coli* cells, promising colonies could be picked after the assay directly from the plate screened. Therefore, there was no need for replica plates during the primary screening of the mutant library. Thus, each round of screening was conducted in a simple and rapid manner. The typical screening of a library of mutant *E. coli* colonies is shown in Supplementary data (Figure S1).

However, care should be taken during the screening procedure to avoid cross-contamination of the colonies. When the screening assay is performed quickly, the cross-contamination is unlikely. We did not observe it in this work and in our earlier search for the color-shifted mutants (Koksharov and Ugarova, 2008). But the plates should not be allowed to be covered by the screening solution for a prolonged time because the colonies would start to partially wash away causing possible cross-contamination. The subsequent heating of the plates at 50°C helps to effectively remove the remaining liquid from the surface of the plates by evaporation. Spraying of the screening solution on the plate was also reported to be an effective approach to prevent cross-contamination (Lockard et al., 2011).

Four consecutive rounds of directed evolution were conducted in order to increase thermal stability of Lml. Mutagenesis was applied to a 785 bp gene region coding for amino acid residues 130–390 out of 548 residues of Lml. The mutant S118C was used as a starting point due to its higher thermostability than that of WT (Koksharov and Ugarova, 2008). The most stable mutant in each cycle was used as a parent for the next round of mutagenesis. The main details and results of the four cycles of directed evolution are summarized in Table I. We aimed for a mutation rate of ~1 amino acid change (two to three base changes) per the region mutated and 30–40% of inactive clones in the library, which is reported to be most convenient for an efficient selection of beneficial mutations (Cirino et al., 2003). The resultant ratio of inactive clones turned out to be slightly higher as seen from Tables I and II and was suitable for the successful selection of thermostable mutants.

During the first cycle of mutagenesis the mutant colonies were screened for *in vivo* bioluminescence activity directly after growing at 37°C. The insufficient stability of WT Lml at these conditions allowed identifying three clones with increased thermostability (Table I) that led to distinctly brighter colonies. These mutants demonstrated similar level of *in vivo* stability, and their *E. coli* colonies generated similar yellow–green bioluminescence compared with orange–yellow light produced by the parent enzyme S118C (Supplementary data, Figure S2) indicating lower pH-sensitivity of these mutants. During the second and third cycles of mutagenesis an additional incubation at 50°C was needed to detect more thermostable mutants. Figure 1 shows a comparison of *in vivo* thermal stability of the mutants obtained during the first three cycles. Three mutants obtained at the third cycle displayed similar bioluminescence brightness after incubation at 50°C, but increasing temperature to 55°C revealed higher stability of the mutants 3T1, 3T2 compared with 3T3. The fourth cycle led
Table I. Results of random mutagenesis and screening for increased thermostability of *Lumicola mingrelica* firefly luciferase

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Number of clones screened</th>
<th>Ratio of active clones (%)</th>
<th>Incubation temperature before screening (°C)</th>
<th>Thermostable mutants identified*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>800</td>
<td>54</td>
<td>37</td>
<td>1T1, 1T2, 1T3</td>
</tr>
<tr>
<td>2</td>
<td>900</td>
<td>52</td>
<td>50</td>
<td>2T1, 2T2</td>
</tr>
<tr>
<td>3</td>
<td>600</td>
<td>65</td>
<td>50</td>
<td>3T1, 3T2, 3T3</td>
</tr>
<tr>
<td>4</td>
<td>1400</td>
<td>65</td>
<td>55</td>
<td>4TS</td>
</tr>
</tbody>
</table>

*The most thermostable mutant in each cycle that was used as a parent enzyme for the following cycle is shown in bold and underlined.

Table II. Nucleotide and amino acid substitutions in evolved thermostable mutants

<table>
<thead>
<tr>
<th>Mutant enzyme</th>
<th>Parent enzyme</th>
<th>Substitutions compared with the parent enzyme</th>
<th>Codon change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1T1</td>
<td>S118C</td>
<td>T213S</td>
<td>acc/agc</td>
</tr>
<tr>
<td>1T2, 1T3</td>
<td>S118C</td>
<td>silent L294</td>
<td>tct/ttg</td>
</tr>
<tr>
<td>2T1</td>
<td>1T1</td>
<td>silent S300</td>
<td>agt/agc</td>
</tr>
<tr>
<td>2T2</td>
<td>1T1</td>
<td>silent P135</td>
<td>tct/gct</td>
</tr>
<tr>
<td>3T1, 3T2</td>
<td>2T1</td>
<td>K156R</td>
<td>aat/aga</td>
</tr>
<tr>
<td>3T3</td>
<td>2T1</td>
<td>A217V</td>
<td>gcg/gta</td>
</tr>
<tr>
<td>4TS</td>
<td>3T1</td>
<td>silent S234</td>
<td>tca/tcg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>silent A330</td>
<td>gcg/gca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E356V</td>
<td>gaa/gta</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E364V</td>
<td>gaa/gta</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E365K</td>
<td>gaa/gaa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C146S</td>
<td>tgt/agg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R211L</td>
<td>cga/cta</td>
</tr>
<tr>
<td></td>
<td></td>
<td>silent R389</td>
<td>cga/ctg</td>
</tr>
</tbody>
</table>

Sequence analysis

DNA sequences of the nine mutants selected during the four cycles of directed evolution were determined (Table II). The resultant mutant 4TS contained eight substitutions compared with WT: S118C (parent enzyme), T213S, K156R, R211L, A217V, C146S, E356K and S364C. The last seven substitutions appeared in the course of directed evolution. The mutants 1T2, 1T3 and the mutants 3T1, 3T2 turned out to be identical. This is most likely the result of the clone duplication at the stage of bacterial transformation. The substitution E356V appeared during the second and third cycles of mutagenesis but did not result in the most stable mutant at each cycle.

Expression and purification of mutant and WT luciferases

WT Lml and the mutant 4TS were expressed using the pETL7 plasmid, and hence, contained additional N-terminal peptide MASK- and C-terminal peptide AKM was changed to the sequence -SGPVEHHHHH-. Average yields of the purified proteins (mg/0.2 l) were 32 mg for WT and 60 mg for 4TS. The 1 ml Ni-IDA column was able to bind ~33 mg of luciferase. The purity of the enzymes was >95% as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. After the purification Lml enzymes were obtained in HB buffer containing 300 mM imidazole, 2 mM EDTA, 1 mM DTT and generally remained fully active for up to 1 month when stored at 4 °C. For the long-term storage the proteins were transferred to GF buffer and stored at ~80 °C. When frozen in GF, luciferase retained full activity for at least 2 years and tolerated several freeze-thaw cycles without inactivation.

In the case of 4TS the addition of DTT was not necessary for the storage of the enzyme at 4 °C. WT luciferase was shown to lose ~70% of activity within 2 weeks when stored in HB buffer containing 300 mM imidazole, 2 mM EDTA without DTT added. The mutant 4TS (1.7 mg/ml) remained fully active under these conditions within 1 month of storage and displayed only 10% decrease in activity after 3.5 months compared with the solution with DTT added.

Biochemical properties

The main catalytic and bioluminescent features of the mutant enzymes are presented in Table III. The properties of the mutant S118C are also included for the comparison. The substitutions in the mutant 4TS led to a 1.9-fold increase in specific activity as well as to 5.7-fold improvement in *K* \textsubscript{m} compared with WT. WT firefly luciferases generally have a temperature optimum of activity at 22–25 °C (Seliger and McElroy, 1964; Mortazavi *et al.*, 2008). *Escherichia coli* colonies harboring WT Lml, which were treated with luciferin solution to induce bioluminescence and then heated from the room temperature to 37 °C, showed a notable decrease of the bioluminescence brightness, and at 50 °C the bioluminescence became almost unnoticeable. On the contrary, the brightness of the colonies harboring mutant 4TS was notably higher when heating the colonies from the room temperature to 37 °C. Upon further heating the colony brightness did not show significant change up to 50 °C, and at 55–60 °C the bioluminescence began to decrease. The higher-temperature optimum of activity was later confirmed for the purified enzyme.

![Fig. 1. *In vivo* bioluminescence of luciferase mutants after incubation of *E. coli* colonies at 50 °C for 40 min.](https://academic.oup.com/peds/article-abstract/24/11/835/1503578/fig1)

Fig. 1. *In vivo* bioluminescence of luciferase mutants after incubation of *E. coli* colonies at 50 °C for 40 min.

to the mutant 4TS. When incubating colonies of mutants 4TS and 3T1 at 55 °C for 40 min the former retained noticeable brightness of bioluminescence while the latter was completely inactivated. Thus, the mutant 4TS showed the highest *in vivo* thermostability among mutants obtained in this study. Moreover, the colonies of 4TS displayed decreased but noticeable bioluminescence after 20 min at 60 °C. At this temperature *E. coli* cells completely lost their viability after 2 min, and so the use of replica plates would be required for the selection of mutants with higher thermostability.

It is noteworthy that *in vivo* bioluminescence of the mutant 4TS showed appreciably higher temperature optimum
value for ATP. The mutant 4TS displayed typical flash-like light emission kinetics with the kinetic profile close to that of WT except for a slightly higher decay time that resulted in a slight increase of the integrated specific activity (Supplementary data, Figure S3).

**Bioluminescence spectra**

Bioluminescence emission spectra of WT Lml and the mutant 4TS were measured over the pH range from 6.0 to 9.0 at 25°C (Table III, Fig. 2). At the pH optimum of 7.8, the mutant 4TS had a wider spectrum in comparison with WT indicating a higher contribution of the red emitter (Fig. 2). WT and 4TS showed the classic red shift (Seliger and McElroy, 1964; Fraga, 2008) of the bioluminescence spectra at acidic pH. Elevated temperatures are known to be another factor leading to the red shift of the firefly luciferase bioluminescence spectra (Seliger and McElroy, 1964). To investigate the effect of temperature on the emission color, bioluminescence spectra were measured at 10, 20, 25, 37 and 42°C (Table III, Fig. 2). At the pH optimum of 7.8, increasing temperature from 25 to 42°C greatly enhanced the contribution of the red emission in the case of both WT and 4TS. Lowering the temperature to 10°C narrowed the spectra of WT and 4TS almost eliminating the contribution of the red emitter in both cases (Fig. 3).

**Thermostability**

First, the thermal stability of 4TS, the mutant S118C and WT Lml was compared at 42°C in Tris-acetate buffer TsB1 (Table III, Fig. 4). This buffer solution is close in composition with the typical reagents for the bioluminescent ATP assay (Moroz et al., 2008). Directed evolution has increased the half-life of Lml at 42°C ~65-fold compared with WT. The starting mutant S118C shows the 1.5-fold increase in stability at these conditions so most of the stabilization is due to the seven substitutions obtained during directed evolution. Then the thermal inactivation of 4TS and WT has been studied in TsB1 in the range of temperatures from 37 to 55°C. Thermal inactivation of WT and 4TS was also studied in the Na-phosphate buffer TsB2 in the same range of temperatures to compare our results with the literature data. The buffer solution similar to TsB2 was used by a number of authors for testing thermostability of luciferase mutants (Kajiyama and Nakano, 1994; White et al., 1996; Kitayama et al., 2003).

At all temperatures studied the thermal inactivation obeyed the first order with the exception of the inactivation in TsB1 buffer at 37°C when both WT and 4TS showed a stage of faster inactivation to 50% (WT) or to 80% (4TS) of activity followed by the stage of slower inactivation (Fig. 4). In the case of WT this bend of the inactivation curve was less pronounced.

The constants of thermal inactivation for different temperatures are presented in Supplementary data (Table S1). The Arrhenius plot for these constants is shown in Fig. 5 (the values for the slow stage are presented for 37°C). At all the temperatures studied 4TS was significantly more stable than WT, but elevating the temperature lowered this difference. For example, the half-time of 4TS at 37°C in TsB1 buffer is 158 times greater than that of WT, but the difference decreases to 65 times at 42°C and to six times at 50°C. As can be seen from the Arrhenius plot, TsB2 buffer causes significant stabilization of both WT and 4TS compared with TsB1 buffer. But the extent of stabilization also decreases with temperature. The stability of WT luciferase at 37°C is 38 times greater in TsB2 buffer compared with TsB1 buffer.
but it decreases to only four times at 50°C. Thus, the higher starting level of luciferase stability leads to a higher degree of stabilization caused by the mutations in the mutant 4TS or by using the stabilizing TsB2 buffer.

It is noteworthy that even in TsB2 buffer the mutant 4TS loses half of its activity within 1.3 min at 55°C, whereas in vivo in E. coli colonies it retained appreciable bioluminescence after 20 min at 60°C. Thus, in vivo microenvironment provides much higher stabilization of luciferase than buffers typically employed.

### Optimum temperature for luciferase activity

The optimum temperature for the activity was estimated for both WT and 4TS (Fig. 6). The activity of WT luciferase reached maximum at 25°C and decreased at higher temperatures. The activity of 4TS reaches a plateau at 37–50°C, which is consistent with the in vivo observations.

Bioluminescence spectra of the firefly luciferase undergo a red shift at elevated temperatures that, given the same light output, leads to a decrease in the bioluminescence intensity measured by the luminometer due to a low and limited sensitivity of the PMT at wavelengths >600 nm. Therefore, in the boundary case of monomodal green and red emission the registered activity for the red bioluminescence would be ≈4.2 times lower (results not shown). This effect is not important in the case of WT luciferase because the significant changes of its spectra begin when its activity is already low (Figs 3 and 6). But in the case of 4TS the correction for the PMT sensitivity was necessary (Fig. 6), otherwise the measured maximum of activity was mistakenly observed at 30°C.

### Discussion

#### Thermostability screening

We used the in vivo screening procedure to identify thermostable mutants of firefly luciferase directly in E. coli colony library. Up to 2000–3000 colonies could be screened on a single 90 mm petri dish. A cycle of random mutagenesis and screening of 1000 colonies typically results in one to two different thermostable mutants. Thus, each round of mutagenesis can be conducted in a simple and rapid manner. The part of the Lml gene coding for amino acid residues 130–390 was targeted for mutagenesis because of the convenient restriction sites and because most reported mutants that enhance the thermostability of firefly luciferases fall within this region. The four cycles of directed evolution have increased the half-life of Lml 65-fold at 42°C in TsB1 buffer. Moreover, although the intermediate mutants were not checked for the catalytic efficiency, the resultant mutant 4TS showed the significant improvement in specific activity and $K_m$ for ATP.

Further stabilization can be achieved by additional cycles of mutagenesis. In this case the use of replica plates will be required at the colony screening step because the incubation temperature will exceed 60°C making the screening procedure lethal for E. coli cells. Also, the remaining C-terminal region (amino acid residues 391–544) of firefly luciferase can be
explored because few thermostable mutants are known for this part of the protein: P407T/G396R (M. Koksharov, unpublished results) and F467R (Law et al., 2006).

**Structural analysis**

The mutant 4TS contained seven 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 II), literature data and their location in the 3D structure of the enzyme (Fig. 7), four of these substitutions are suggested to be mainly responsible for the increase in thermal stability: R211L, A217V, E356K and S364C. In the previous studies the mutations of the residues A217 (Kajiyama and Nakano, 1993) and E356 (White et al., 1996) were shown to significantly increase the thermostability of firefly luciferases. Mutations of the residues R211 and S364 are reported for the first time. The thermostabilizing effect of the substitutions R211L, S364C and S364A can be attributed to the improvement of the internal hydrophobic packing by the substitution of the non-conservative buried polar residues by the hydrophobic ones (Fersht and Serrano, 1993). The mutants C146S (Modestova et al., 2011) and S118C were shown to cause a slight increase in thermal stability (1.3- and 1.5-fold, respectively) at 42°C. Therefore, they may contribute in part to the high thermal stability of 4TS. The surface mutation C146S is also known to increase the resistance to oxidative inactivation (Modestova et al., 2011) and can explain the increased storage stability of 4TS in the absence of DTT compared with WT. The mutants T213S/

S364C and S364A displayed similar *in vivo* properties. Thus, it is unlikely that the substitution T213S is accountable for the increase in stability. The substitution of the surface residue 156 from positively charged Lys to Arg is also unlikely to affect thermostability.

Figure 7 shows the positions of the substitutions of the mutant 4TS in the 3D-structure model of Lml generated by homology modeling using the structure of Lcl (Koksharov and Ugarova, 2008). The luciferase structure consists of three distinct subdomains. The Subdomains A and B compose the large N-terminal domain of firefly luciferase while the Subdomain C is the smaller C-terminal domain. The two domains are connected by a flexible linker loop (Nakatsu et al., 2006). All four presumable key thermostabilizing substitutions (R211L, A217V, E356K and S364C) are located in the second subdomain of firefly luciferase. Although the larger part of the mutated region belongs to the second subdomain (198 amino acid residues), the fact that thermostabilizing mutations were not found in the last 62 amino acid residues of the first subdomain is consistent with the findings of Frydman et al. (1999) and coworkers who had investigated the unfolding of firefly luciferase by chemical denaturation with subsequent protease treatment. The authors have shown that the fragments comprising of residues 1–190 and 422–544 possess high intrinsic stability. These fragments mainly correspond to the Subdomains A and C of firefly luciferase. The middle Subdomain B (192–435) was shown to be significantly less stable and was the first to unfold under denaturing conditions. Thus, it may be...
concluded that the stability of the second subdomain is the main factor that determines the stability of the firefly luciferase protein. Therefore, most of the thermostabilizing mutations should be located in the second subdomain or in an interface between the second subdomain and the other two subdomains. It is noteworthy that almost all thermostable mutants reported in the literature are located in this part of the luciferase structure. The mutant S118C is located at the interface between the Subdomains A and B.

Due to the presence of the eight substitutions in 4TS it is unclear as to what mutations contribute to the significant decrease of the \( K_m \) for ATP, and further mutagenesis experiments are required to definitely answer this question. In contrast to the other substitutions in 4TS, the residue S364 is located near the adenosine moiety of ATP (Fig. 7). Therefore, the substitution S364C may be one of the main factors responsible for the decrease of \( K_m \) for ATP. The substitution S118C causes a slight decrease of \( K_m \) for ATP (Table III). It is located away from ATP molecule near the residues participating in binding of \( Mg^{2+} \) and the phosphate moiety of ATP. The substitutions A217L, E354K in Ppl (Tisi et al., 2002) and C146S in Lml (Modestova et al., 2011) did not change the \( K_m \) for ATP according to the literature, so the corresponding mutations are less likely to contribute to the improvement of \( K_m \) of the mutant 4TS.

**Bioluminescence spectra**

Bioluminescence spectrum is a key feature of luciferase enzymes. Most firefly luciferases demonstrate highly pH-sensitive bioluminescence spectra that undergo a large shift from the green to red region on lowering pH from alkaline to acidic. Red shifts are also observed at elevated temperatures and increased concentration of divalent heavy metal ions (Seliger and McElroy, 1964). This red shift is attributed to the switching between two different molecular forms of the product (green and red emitters) (Fraga, 2008). In some cases mutants that significantly increase thermostability also lead to a low pH-sensitivity of the bioluminescence spectra (Kitayama et al., 2003; Law et al., 2006). The thermostable mutant 4TS displays broadened and pH-sensitive bioluminescence spectra, thus confirming that generally thermostability and pH-sensitivity are independent. The broadened bioluminescence spectrum of 4TS and its narrowing at low temperatures indicate that the microenvironment of the emitter in the active site became more flexible in spite of the increase of the overall protein stability.

The broadening of the spectra may be attributed to the effect of the substitutions A217V and E354K, which were shown to increase the ratio of red emitter in case of Lml (Koksharov and Ugarova, 2011) or Hpl (Kitayama et al., 2003), Ppl (White et al., 1996) and *Lampyris turkestanicus* luciferase (Alipour et al., 2009) respectively. In contrast, the mutation S364C counteracts the broadening of the spectra by decreasing the contribution of the red emitter (Supplementary data, Figure S2).

**Comparison of the thermostability of the mutant 4TS with the previous studies**

The mutant 4TS developed in this study appears to be one of most stable firefly luciferase variants available. Its stability was significantly higher than that of the recently developed multi-point thermostable mutant of Ppl (half-time of 129 h for Lml versus 11.5 h for Ppl at 37°C in the buffers with a moderate ionic strength), which represents the thermostable version of the most widely used firefly luciferase (Branchini et al., 2007). The stability of 4TS also surpasses the stability of the thermostable mutant E356R/V368A of Hpl (half-life of 33.4 versus 3.5 h at 45°C in the buffer TsB2) (Kitayama et al., 2003) and the mutant T217V of Lcl (half-life of 44 versus 28 min at 50°C in the buffer TsB2) (Kajiyama and Nakano, 1993). However, the mutant *Luciola lateralis* luciferase with the substitution A217L shows greater stability (half-life of 125 min at 50°C in the buffer TsB2) than the mutant 4TS owing to the fact that WT *L. lateralis* luciferase is naturally the most thermostable among WT firefly luciferases (Kajiyama and Nakano, 1994). The 28-point mutant of *P. pennsylvanica* luciferase developed in Promega Corp. (Wood and Hall, 1999) still remains the most thermostable variant of firefly luciferase to date. The conduction of additional cycles of directed evolution and the introduction of additional mutations is needed for a further increase of Lml thermostability.

**Conclusions**

We demonstrated that the *in vivo* directed evolution strategy is a simple and efficient approach to increase firefly luciferase thermostability without sacrificing catalytic efficiency. In fact, the resultant mutant displayed superior catalytic properties: increased specific activity, lower \( K_m \) for ATP and increased temperature optimum.

In typical applications, for example, ATP measurement or as a reporter gene, firefly luciferase is usually used at temperatures up to 37°C. The mutant 4TS retains 70% activity after 2 days of incubation at 37°C. Thus, its stability is sufficient for most common *in vivo* and *in vitro* applications. The improved protein yield, specific activity and catalytic efficiency of the mutant 4TS make it an efficient tool for ATP determination (Ugarova et al., 2010). The increased temperature optimum of 4TS can be advantageous for *in vivo* imaging and high-temperature applications. The non-lethal *in vivo* screening approach described in the present study can potentially be adapted to other beetle or non-beetle luciferases when the increased level of thermal stability is desirable.

**Supplementary data**

Supplementary data are available at PEDS online.

**Acknowledgements**

The authors thank Lothar Nofer (Berthold Detection Systems GmbH, Germany) and Graham Sperrin (ET Enterprises Ltd, UK) for the data on the PMT used in FB12 luminometer and its spectral response curve. We thank Dr Ekaterina I. Dementieva (Engelhardt Institute of Molecular Biology RAS, Russia) for a careful reading of the manuscript and useful suggestions.

**Funding**

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

References