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SCIENTIFIC REVIEW

Luciola mingrelica Firefly Luciferase: Historical Aspect

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Abstract—This review presents the history of research on the luciferin–luciferase system of fireflies *Luciola mingrelica* at the Division of Chemical Enzymology, Department of Chemistry, Moscow State University, which began in the mid-1970s at the initiative of the first head of the Department, Professor I.V. Berezin. Based on the study of the kinetics of enzymatic oxidation of luciferin, a kinetic scheme of the reaction was proposed, according to which in an aqueous solution the luciferase reaction is a nonstationary enzymatic process and the turnover of the enzyme is very small due to the slow dissociation of the enzyme–product complex. Analysis of the bioluminescence and fluorescence spectra of the reaction product oxyluciferin and its analogs led to the conclusion that keto–enol tautomers of the phenolate forms of oxyluciferin (ketone, enol and enolate ion) are the most likely emitters in the luciferin–luciferase system of fireflies. Native luciferase preparations have been shown to contain phospholipids, whose removal leads to a decrease in the activity and stability of the enzyme. At the beginning of the 1990s, *L. mingrelica* luciferase was cloned. The enzyme in the primary sequence turned out to be close to other luciferases of the genus *Luciola*, cloned in Japan (more than 80% homology), but differed from the previously studied luciferase from American *P. pyralis* fireflies (67% homology). Using methods of random and site-specific mutagenesis, a library of mutant forms of *L. mingrelica* luciferase with altered bioluminescence spectra (green and red luciferases) was created. Thermostable mutants of luciferase were obtained by the method of directed evolution, and in particular, a highly active and thermostable mutant (4TS), on the basis of which an ATP-reagent was developed, which is still widely used in bioluminescent analysis by many researchers in Russia. Genetic engineering, computer modeling and site-specific mutagenesis methods have been used to clarify the role of the dynamic structure of the enzyme in the complex, three-stage oxidation of the luciferin. It has been shown that the emitter (electronically excited oxyluciferin) is an intramolecular label in the enzyme's active site. The superposition of two or three emitter forms fixed in the bioluminescence spectra indicates the coexistence of various conformational forms of luciferase in the reaction medium, which are in dynamic equilibrium.

Keywords: bioluminescence, firefly luciferase, *Luciola mingrelica*, luciferin, ATP, oxyluciferin, emitter, kinetics, thermal stability, mutagenesis

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The bioluminescence of fireflies has fascinated people since ancient times, but only at the end of the 19th century it was established that this phenomenon is the result of the oxidation of an organic compound (the luciferin substrate) by the enzyme luciferase [1]. The modern stage of studying firefly luciferases began in the late 1940s, when a young American scientist D. McElroy established that the necessary cosubstrate in this reaction is ATP, the most important intracellular metabolite determining the energy status of the organism [2]. The firefly luciferin–luciferase system has formed the basis of a rapid, specific, and highly sensitive method for ATP determination. Research on the mechanism of the action of luciferase of American firefly *Photinus pyralis* has expanded [3]. In our country, some researchers obtained extracts from Russian fireflies and used them to measure ATP concentrations. In the mid-1970s, on the initiative of Prof. I.V. Berezin at the Division of Chemical Enzymology, Department of Chemistry, Moscow State University, the kinetics and mechanism of the action of firefly *Luciola mingrelica* luciferase, inhabiting Russia, began to be studied and the development started of bioanalytical systems based on it for practical use in biotechnology, ecology, and medicine. This review examines the main achievements of Russian scientists on this issue.

Native Firefly Luciferase of Luciola mingrelica: Kinetics and Mechanism of Action

Firefly luciferase is of interest not only as a specific reagent for determining microquantities of ATP but also as a unique biocatalyst for the highly efficient bioconversion of energy into light. *L. mingrelica* luciferase, as it turned out, is like *P. pyralis* luciferase in terms

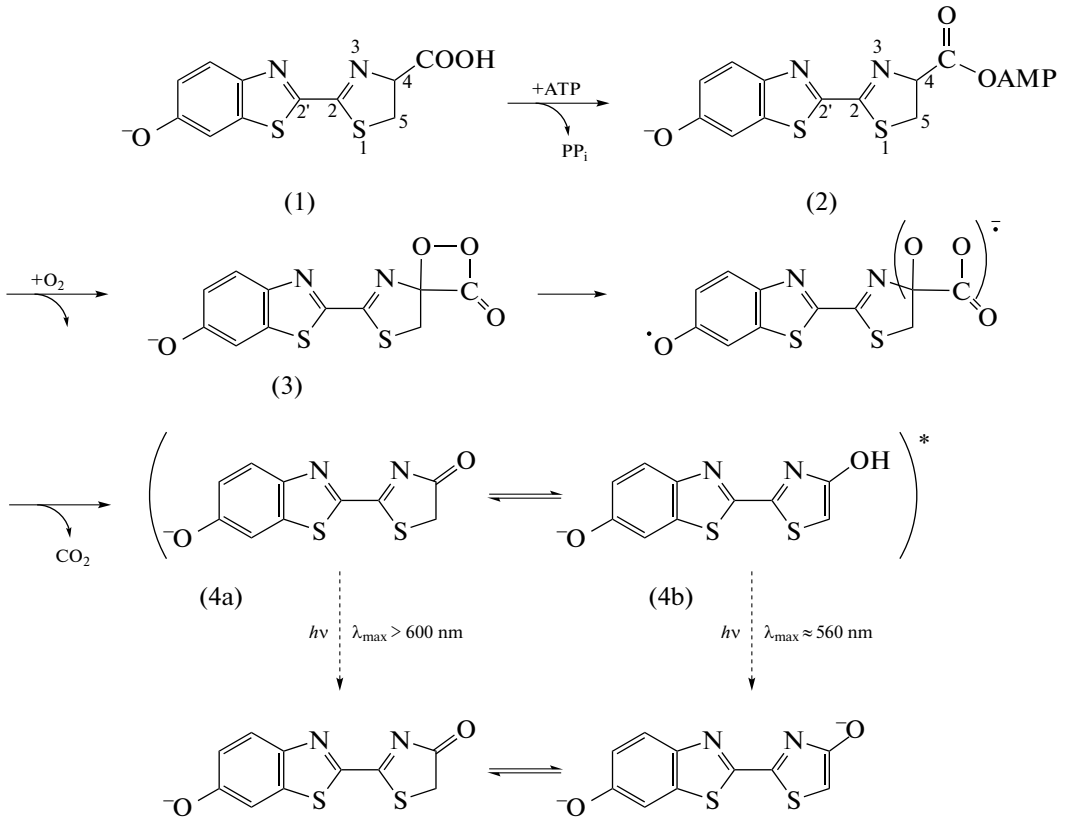

Fig. 1. Mechanism of the oxidation reaction of luciferin.

of its biochemical and kinetic properties [4]. Bioluminescent oxidation of luciferin is a complex multistage process that proceeds through the formation of a triple enzyme–substrate complex, then through a series of intermediate stages an electronically excited product is formed, during the transition of which to the ground state with a high quantum yield, the emission of visible light is observed [5] (Fig. 1).

The kinetics of the enzymatic oxidation of firefly luciferin in the presence of *L. mingrelica* luciferase was studied in a 0.1 M Tris-acetate buffer solution over a broad time range (from milliseconds to hours) at different concentrations of luciferin and ATP [6]. As a result, a kinetic scheme that includes four sequential-parallel stages of the process was proposed (Fig. 2). The intensity of bioluminescence characterizes the rate of emission of light quanta and it is proportional to the concentration of the intermediate product (EP1):

$$I = d[\mathrm{N}]/dt = k_3[\mathrm{EP1}].$$

In aqueous solutions, the kinetic curves of bioluminescence have the form of curves with a maximum. The Michaelis–Menten equation is formally satisfied only at the maximum point of the kinetic curve [7]. A feature of the luciferase reaction is that the degree of conversion of substrates is quite low, and the decrease in the intensity of luminescence after reaching the maximum is not explained by either the consumption of substrates or the inhibition of the enzyme by the product or substrates of the reaction. The analysis of the kinetics of bioluminescence in the initial period of the reaction (0.5–30 ms) using the "stopped jet" method and the analysis of the integral kinetic curves (up to 99% of the luminescence decline) [6] showed that the kinetic curve has an induction period (1.0–3.0 ms), followed by a monoexponential increase in luminescence to the maximum point (0.15–0.40 s) and a subsequent long-term decline in intensity to almost zero over several hours (Fig. 3). The induction period decreases with the increasing concentration of the substrates and enzyme and, therefore, is determined by the first stage of the process—the formation of the enzyme–substrate complex (Fig. 3a). The second stage (transformation of the ternary complex ES_1S_2 in EP_1 with $k_2 = 20\ s^{-1}$) limits bioluminescence until maximum luminescence is reached. The third stage (decay of bioluminescence) is described by a combination of several exponentials (k_3 and k_4), whose parameters were determined by the numerical analysis of the complete kinetic curves. The k_4 value is about a tenth of the value of k_r and k_3; therefore, in subsequent cycles of the enzymatic reaction, the rate of formation of the electronically excited product is an order of magnitude lower, since it is limited by the rate of

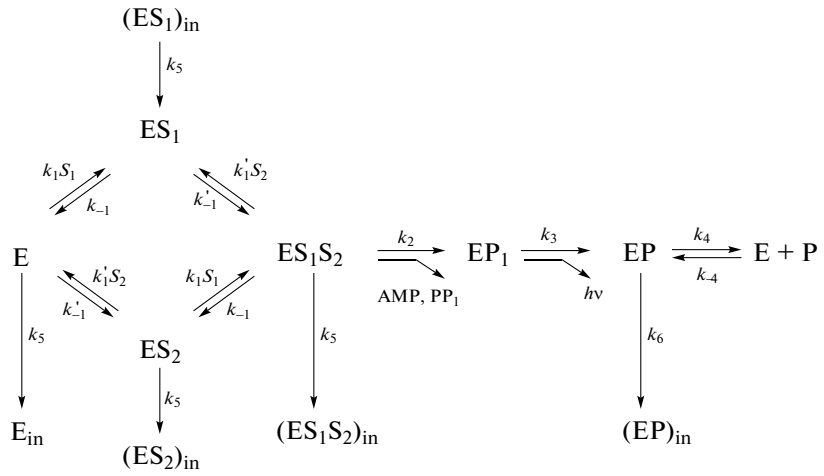

Fig. 2. Kinetic scheme of reactions of enzymatic oxidation of luciferin.

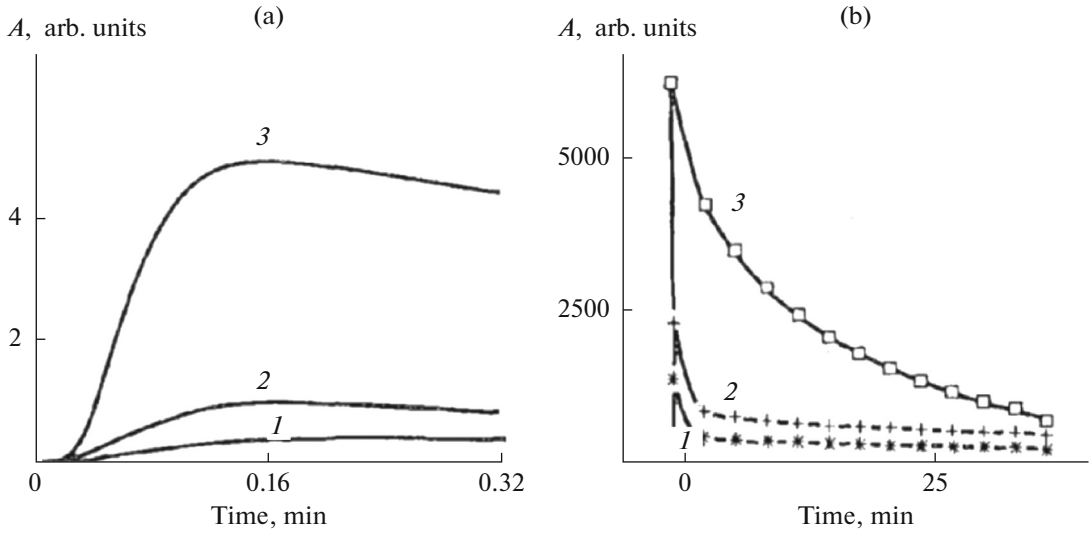

Fig. 3. Kinetic curves of bioluminescence in the firefly luciferin–luciferase reaction in second (a) and minute (b) time intervals. Conditions: 0.1 M Tris-acetate buffer solution containing 2 mM EDTA; 10 mM $MgSO_4$; pH 7.8; 1 mM luciferin. ATP concentration, mM: (*1*) 0.05; (*2*) 0.1; (*3*) 1.

regeneration of the active enzyme. However, due to the parallel inactivation of the enzyme (Fig. 2, reactions *5* and *6*), this rate does not remain constant, but gradually decreases.

The above-mentioned mechanism demonstrated the inconsistency of the previously proposed hypothesis [8] about the existence of two catalytic centers (fast and slow) in firefly luciferase, which provide high (at the maximum) and low (in decline) luminescence intensity. Thus, the luciferase reaction serves as an example of a nonstationary enzymatic process, where the turnover of the enzyme is very small due to the slow dissociation of the enzyme–product complex and the commensurate rate of inactivation of the enzyme and its complexes with the substrates and product. Identification of the rate-limiting stages of the bioluminescence process also points to practical ways to increase the efficiency of luciferase. The use of various stabilizing additives, effectors that accelerate the regeneration of the native enzyme, leads to a significant increase in the total yield of the electronically excited product, i.e., the total yield of light quanta, and to the creation of bioluminescent reagents with a constant glow.

The bioluminescence spectra for the luciferase reaction were obtained in the pH range of 5.6–8.8 [9]. (Fig. 4). The shape of the spectrum in the pH range of 7.0–8.8 did not change. At pH < 7.0, the spectrum maximum shifts to the long-wave region. The enzyme activity was maximal at pH 7.8 and decreased with

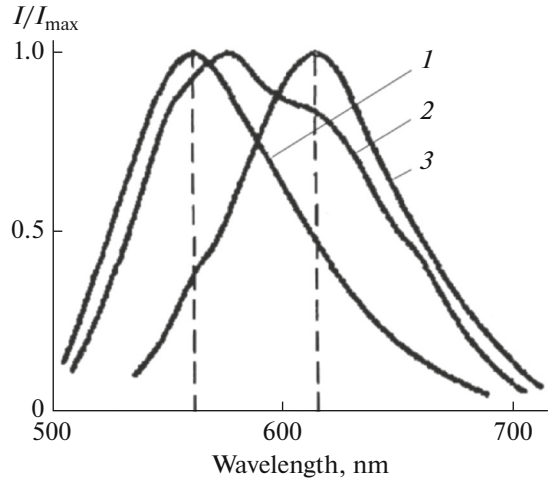

Fig. 4. Bioluminescence spectra for the native bioluminescent system of *L. mingrelica* fireflies at pH 7.0 (*1*); 6.7 (*2*); 5.6 (*3*).

both decreasing and increasing pH, which is explained by the acid-base properties of the protein and the decrease in the quantum yield of bioluminescence. The shift of the spectrum to the long-wave region at pH < 7.0 is explained by a change in the properties of the emitting particle at these pH values [9].

The reaction product—oxyluciferin (LO) in a singlet electronically excited state [10]—is such an emitting particle. The transfer of a molecule to an electronically excited state can occur because of the absorption of energy from electromagnetic radiation or ionizing radiation, as well as a result of highly exothermic chemical or biochemical reactions. On excitation, these pathways are fundamentally different. However, in the condensed phase, the differences are eliminated due to the rapid (within picoseconds) establishment of thermal equilibrium; thus, the fluorescence of oxyluciferin in solution was considered as a model of bioluminescence in the luciferin–luciferase system of fireflies [11–13]. The steady-state and subnanosecond time-resolved fluorescence of oxyluciferin (LO) and its structural analogs were studied: luciferin (LH_2), 6'-methoxyluciferin ($MeOLH_2$), and 2-cyano-6-hydroxybenzothiazole (BT) in aqueous (pH 1–10) and ethanol solutions. There are data in the literature on the fluorescent properties of LO and its analogs [14–16], but a detailed study of their fluorescence in different environments over a wide pH range has not been conducted. The fluorescence spectra of oxyluciferin have previously been obtained only in organic media [17] due to its instability in aqueous solutions in the presence of oxygen. Therefore, aqueous oxyluciferin solutions were prepared by mixing a buffer solution previously degassed in vacuum with the minimum volume of an alcoholic LO solution, and all measurements were performed under anaerobic conditions.

The analysis of the obtained data led to the following conclusions. Oxyluciferin can exist in a solution in six forms [12] (Fig. 5). Phenolic forms (I–III) exist in nonpolar media or at very low pH values and fluoresce in the blue region of the spectrum (λ_{max} = 450 nm). Blue bioluminescence was not observed in the bioluminescence spectra. In aqueous solutions for phenolate forms (IV–VI), a yellow-green color (λ_{max}= 550–570 nm) or red (λ_{max} = 620 nm) fluorescence is observed. The $MeOLH_2$ and BT, which do not have a keto group, do not fluoresce in the red region, and 5,5-dimethyloxyluciferin, which is not capable of keto-enol tautomerism, has a fluorescence maximum only in the red region. Therefore, the keto form (VI) is a red emitter, and the enol (V) and enolate dianion (IV) are yellow-green emitters. Bioluminescence spectra depend on the structure of luciferase [18, 19], whose protein globule creates a microenvironment of the chromophore, stabilizing various forms of oxyluciferin.

The localization of firefly luciferase to peroxisomes [20, 21] indicates membrane activity of this enzyme. In relation to this, the influence of lipids on the luciferase reaction was studied in detail. It turned out that a highly purified enzyme isolated from natural raw materials contains a certain amount of phospholipids and neutral lipids [22]. Luciferase spontaneously integrates into multilamellar phosphatidylcholine liposomes, which is accompanied by a 20-fold increase in enzyme stability [23]. The incorporation of luciferase in the bilayer of liposomes [23] or in hexameric structures of reverse micelles [24] leads to a change in the kinetics of bioluminescence: the maximum intensity of bioluminescence and the duration of constant luminescence increase. Therefore, the specific interaction of luciferase with membrane-like structures increases the yield of the electronically excited product. The removal of lipids completely inactivates the enzyme [25]. The addition of phosphatidylcholine to the delipidization medium results in reactivation of the enzyme [26] (Fig. 6). Only choline-containing phospholipids (lysophosphatidylcholine, sphingomyelin, phosphatidylcholine) have a reactivating effect on delipidated luciferase [27].

Immobilization of Native Firefly L. mingrelica Luciferase and Application of Immobilized Enzyme

The labor-intensive method of purifying the native enzyme and its low stability in aqueous solutions complicated its use in analysis; therefore, methods for immobilizing *L. mingrelica* luciferase directly from the firefly extract on various powder and film carriers were developed. The most active and stable preparations of immobilized luciferase were obtained using polysaccharide carriers (BrCN-activated sepharose) [28] and cellophane films specially treated to increase their surface area [29, 30].

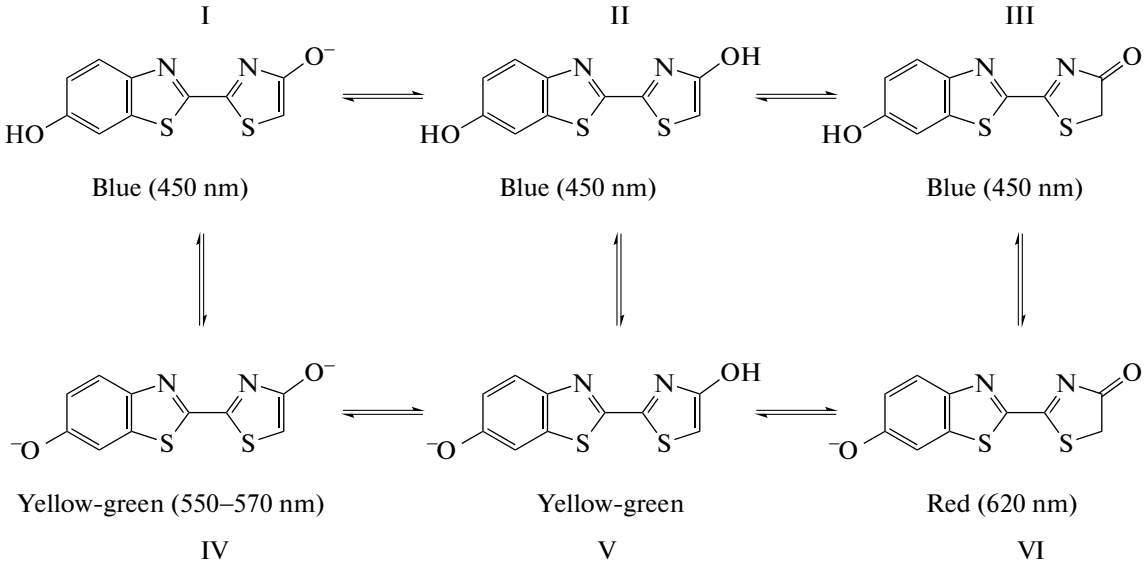

Fig. 5. Different forms of oxyluciferin: (I)–(III) phenolic; (IV)–(VI) phenolate; (I), (IV) enolate; (II), (V) enolic; (III), (VI) ketone.

The ATP-metry is a highly sensitive, rapid, and universal method for biomass determination. ATP is found in all living cells: in the cells of plants, animals, microorganisms, and humans. The ATP content in bacteria ranges from 1 to 10 μg/g dry weight of biomass depending on the type of microorganism and their physiological state. The sensitivity of the bioluminescent method for the determination of ATP is ~10^{-18} moles of ATP in the measured mixture. The detection limit can reach ~500 cells or even less without preliminary sample enrichment. After cell death, the ATP content drops sharply within a few seconds; thus, measuring ATP allows us to determine the content of living cells, in contrast to methods based on measuring other indicator metabolites. The ATP content is proportional to the number of cells in the sample; therefore, the bioluminescent ATP-metry method has become the basis of the so-called "rapid microbiology." The highly active, stable preparation Immolum, based on the immobilized extract of fireflies *L. mingrelica*, was used to determine the microbial biomass [31, 32], ATP concentration, and the activity of enzymes synthesizing or degrading ATP [33]. The bioluminescent method for determining the activity of creatine phosphokinase [34, 35] allowed us to determine this enzyme, which is diagnostically important for the early detection of myocardial infarction, with a lower limit of 1.0 ± 0.2 IU/L. Methods for obtaining coimmobilized three-enzyme systems: firefly luciferase, pyruvate kinase, and adenylate kinase have been developed [36]. Their use allowed us to measure the intracellular content of the adenine nucleotides responsible for the energy state of the cell and to obtain valuable information about the influence of external conditions on intracellular metabolism [37]. The drug Immolum was also used to control the bacterial contamination of raw milk [38] and meat [39], as well as to determine the biocontamination of technological materials [40], and to quickly assess the sensitivity of microflora to antibiotics directly in purulent wounds of patients [41] and in septic blood [42].

Cloning of Firefly L. mingrelica Luciferase

As soon as the first publications on the cloning of firefly *P. pyralis* luciferase appeared in the mid-1980s,

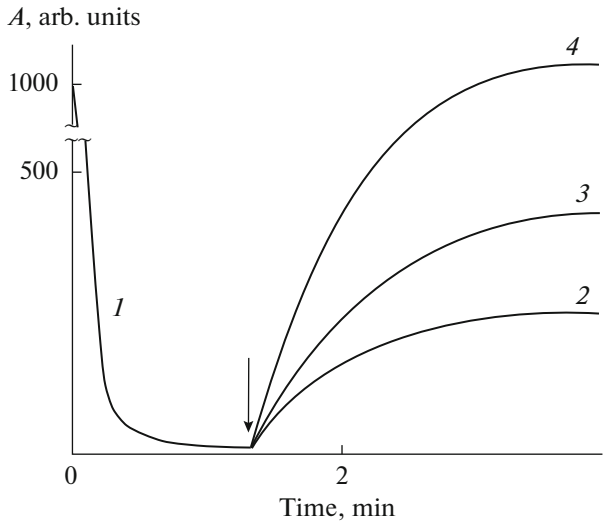

Fig. 6. Inactivation of firefly luciferase with sodium deoxycholate (0.5 mg/mL) (*1*) and reactivation with phospholipid additives (1 mg/mL): phosphatidylcholine (*2*), sphingomyelin (*3*), lysophosphatidylcholine (*4*).

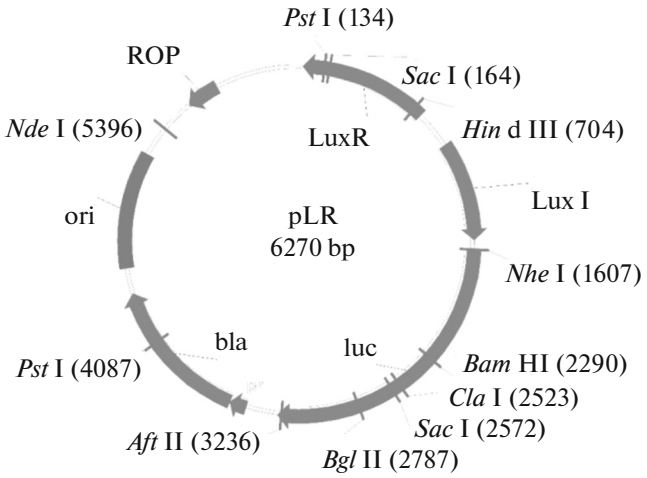

Fig. 7. Plasmid based on the bacterial luciferase expression system (luc, *L. mingrelica* firefly luciferase gene (1615 base pairs); bla, ampicillin resistance gene).

Moscow State University also started to work on this topic. The method for obtaining mRNA was optimized, and firefly *L. mingrelica* mRNA preparations were obtained. The high level of activity of these preparations was demonstrated using frog oocytes and reticulocyte homogenate as an example. Incubation of mRNA in these media resulted in the synthesis of active luciferase: bright bioluminescence was observed upon the addition of luciferin. A manuscript on the production and properties of *L. mingrelica* luciferase mRNA was accepted for publication in the spring of 1987, but the article was published after the death of our coauthor, Prof. I.V. Berezin [43].

For further work on cloning, we accepted the offer of our American colleagues from Texas A@M University, where the laboratory of Prof. T. Baldwin was actively engaged in the genetic engineering of bacterial luciferase and had all the necessary equipment and reagents for genetic engineering studies. Using *L. mingrelica* luciferase mRNA, its cDNA was obtained. The bacterial luciferase gene was cut from the pJGR plasmid and the luciferase gene of the firefly *L. mingrelica* was inserted in its place. In this case, the bacterial luciferase promoter system was used as a promoter (Fig. 7). Thus, a producer of recombinant *L. mingrelica* firefly luciferase with high expression levels was created [44].

The *L. mingrelica* luciferase molecule contains 548 amino acid residues. The amino acid sequence homology with luciferase from American fireflies *P. pyralis* is 67%. The comparison of amino acid sequences of *L. mingrelica* luciferase and luciferase from Japanese fireflies *L. cruciate* and *L. lateralis* showed that about 80% of the residues of these luciferases of the genus *Luciola* are strictly conservative.

Later, pETL4 and pETL7 plasmids were constructed based on the pET series, which encode luciferase containing a 6xHis-tag at the N- and C-terminus, respectively. The use of pETL4 and pETL7 plasmids instead of the previously used pLR plasmid (Fig. 7) allowed us to reduce the duration of expression by half and the purification time from 3 days to 4 h. At the same time, the specific activity of luciferase was doubled, and the enzyme yield increased by a factor of 2.5. The comparison of the two obtained luciferase forms showed that the enzyme containing the C-terminal 6xHis-tag has a higher similarity to the original enzyme in terms of spectral properties and stability. Recombinant *L. mingrelica* luciferase un subsequent studies successfully replaced the natural enzyme and made it possible to obtain new recombinant forms of *L. mingrelica* luciferase with the given properties using site-specific and random mutagenesis methods.

Model of the Spatial Structure of Luciferase and Its Complexes with Substrates

The spatial structure of the unliganded form of firefly *P. pyralis* luciferase was published in 1996 [45]. The enzyme molecule was shown to consist of two easily distinguishable domains: a large N-domain (residues 1–436) and a small C-domain (residues 440–544). The domains are connected to each other by a flexible, disordered polypeptide loop (residues 435–441) (Fig. 8). The computer analysis showed that the spatial structure of *L. mingrelica* luciferase was almost indistinguishable from the structure of *P. pyralis* luciferase. Based on the crystallographic analysis of unliganded luciferase, it was difficult to draw conclusions about the localization of the active site of the enzyme. Due to the high degree of homology of these luciferases, it was difficult to select from the many conserved motifs those that could participate in substrate binding.

In 1997, the results of a crystallographic study of the adenylating subunit of gramicidin-S-synthetase (hereinafter, referred to as synthetase) in complex with its substrates AMP and L-phenylalanine were published [46]. The spatial structure of the synthetase turned out to be very similar to the structure of luciferase. This was expected, since both enzymes perform the same function: adenylation of the carboxyl group of the substrate using ATP. Their amino acid sequences have some weak homology. Using the coordinates of the unliganded luciferase [45] and the enzyme–substrate synthetase complex [46], we constructed a model of the luciferase–ATP–luciferin complex [47], assuming that when substrates bind to luciferase, significant conformational changes occur in the protein molecule, leading to an orientation of the two luciferase domains similar to that found in the structure of the AMP–phenylalanine synthetase complex (Fig. 9). It was shown that only the orientation of the domains change, but the rigidity of those polypeptide loops that are directly in contact with the substrates also increases. The Lys529 residue, required for catalysis, is brought into contact with substrates only

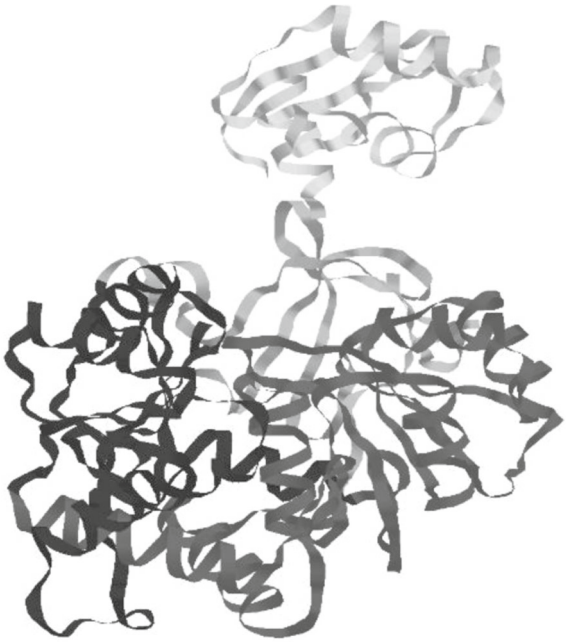

Fig. 8. Spatial structure of unliganded *P. pyralis* luciferase.

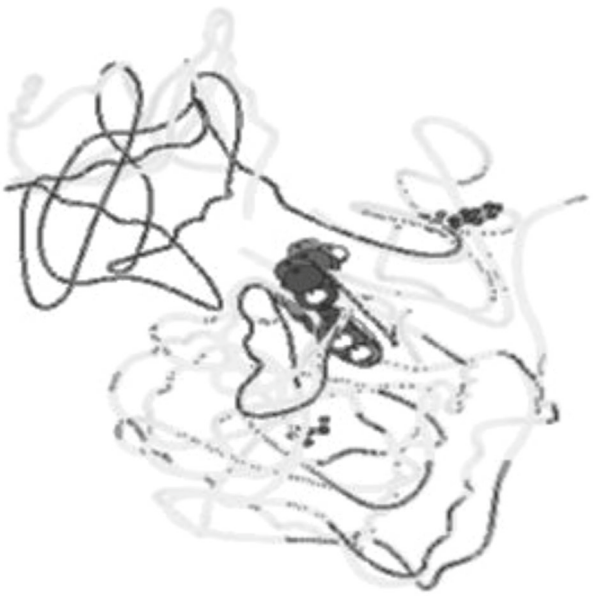

Fig. 9. Model of the *L. mingrelica* luciferase complex with substrates shown as CPK models (nonligand luciferase, gray ribbon; enzyme–substrate complex, black ribbon).

when the domains are rotated. The proposed model is in close agreement with the data on the physicochemical properties of luciferase and its complexes with substrates. It was subsequently successfully used to determine the effect of mutations on the properties of the enzyme.

Construction of the Library of L. mingrelica Mutant Luciferases with Altered Bioluminescence Spectra

As mentioned above, firefly luciferases are characterized by a strong dependence of the bioluminescence spectrum on pH (Fig. 4). As a result of the random mutagenesis of the first 225 residues of *L. mingrelica* luciferase 31 mutants that had an altered bioluminescence color and retained noticeable activity were found. Seven mutants were sequenced [48]. Substitutions of Phe16Leu or Ala40Ser were shown to significantly reduce the pH sensitivity of the firefly *L. mingrelica* luciferase bioluminescence spectrum. For the first time, a single substitution (Tyr35Asn or Tyr35His) was discovered, as a result of which the bioluminescence spectrum of firefly luciferase remained practically unchanged in the pH range of 6–8 (Fig. 10).

The formation of the microenvironment of oxyluciferin in the active center and the implementation of green (or red) bioluminescence are determined by the structure of the protein environment of the emitters, the degree of its polarizability, and the orientation and mobility of the key amino acid groups, i.e., the balance

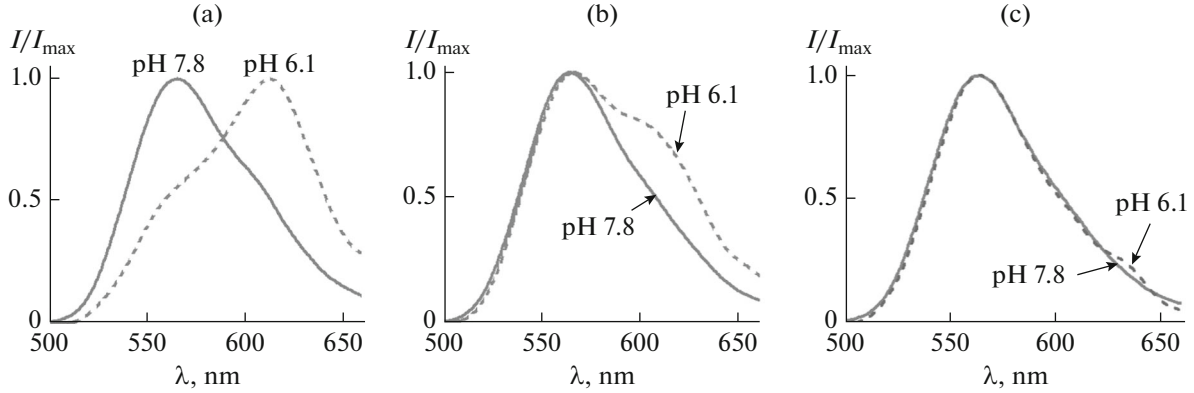

Fig. 10. Bioluminescence spectra of the original luciferase (a), Phe16Leu and Ala40Ser mutants (b), Tyr35Asn and Tyr35His mutants (c) at different pH values.

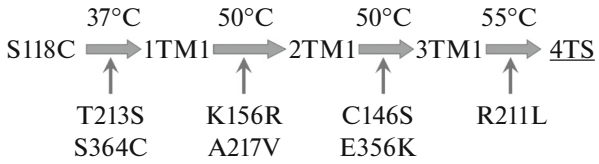

Fig. 11. Scheme for obtaining a thermostable mutant of *L. mingrelica* luciferase using the directed evolution method.

of interactions of many amino acid residues in a large region of the enzyme. The literature describes many mutations distant from each other, which directly or indirectly disrupt the necessary interactions and lead to an increase in the proportion of red bioluminescence [49–51], as well as mutations that stabilize the structure of the active center and reduce the dependence of the bioluminescence spectrum on external conditions [52, 53].

The analysis of the crystal structures of complexes of the original and mutant firefly luciferases (with the Ser286Asn substitution, leading to red bioluminescence) with (DLSA), an analog of the intermediate product of the luciferase reaction (complexes E"DLSA and mE"DLSA) [54] showed that in the E"DLSA complex the active center is in a "closed" conformation. This results in a rigid microenvironment of oxyluciferin and the formation of a green emitter. In the mE"DLSA complex, an "open" conformation of the active center is observed with a less rigid environment of the electronically excited product, upon the transition of which to the ground state, red bioluminescence is observed [54].

The Tyr35 residue is conserved in all firefly luciferases. It borders the 233–237 loop, whose position is important for maintaining the closed conformation of the luciferase active site, which is necessary for the implementation of green bioluminescence. It is quite possible that a decrease in pH leads to a less rigid (open) conformation of the active center of the enzyme, which causes a shift in the bioluminescence spectrum to the red region. When the bulky aromatic residue Tyr35 is replaced by smaller Asn or His residues, the tight packing near 35 and 225 residues becomes more stable and loop 233–237 maintains its position even when the pH decreases; therefore, the closed conformation is not disrupted [48].

Construction of the Library of L. mingrelica Mutant Luciferases with Increased Thermal Stability

Using random mutagenesis by error-prone polymerase chain reaction, a search for *L. mingrelica* luciferase mutants with increased thermal stability was performed [55, 56]. According to the published data [57], the second subdomain of the XhoI-BglII enzyme (395–1180 bp) is significantly more labile than the other two subdomains in the large N-domain of luciferase, which is the main factor in the insufficient stability of luciferase. Therefore, this region of the gene was chosen for mutagenesis. Methods for screening libraries of luciferase mutants in *E. coli* colonies, allowing for the rapid and efficient selection of mutants with increased thermal stability, were developed. The ligation product obtained after random mutagenesis was transformed into *E. coli* XL1blue cells. Cells were grown overnight at 37°C on LB plates containing 100 μ/mL ampicillin. To select more thermostable mutants, cells were incubated at elevated temperatures, which led to the inactivation of insufficiently stable luciferase mutants, and then the bioluminescence of the colonies in vivo was recorded. In the first cycle, screening was performed immediately after cell growth at 37°C; in the second and third cycles, screening was performed after incubation for 40 min at 50°C; and in the fourth cycle, screening was performed after 40 min at 55°C. After the fourth cycle, the 4TS mutant was obtained, whose colonies retained noticeable luminescence even after 20 min of incubation at 60°C (Fig. 11).

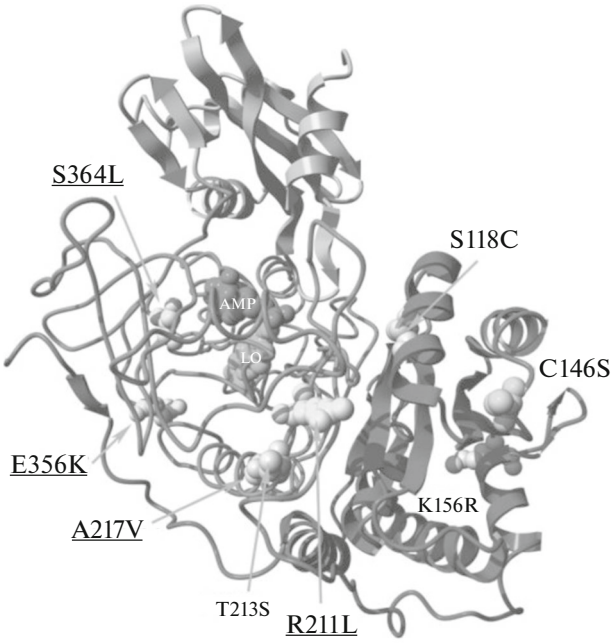

Fig. 12. Localization of mutations in thermostable *L. mingrelica* luciferase (4TS).

Thus, as a result of four consecutive cycles of random mutagenesis, an *L. mingrelica* firefly luciferase mutant with eight substitutions, whose stability increased 66-fold at 42°C, was obtained. The increase in the thermal stability of the mutant enzyme was mainly due to the R211L, A217V, E356K, and S364C substitutions (Fig. 12).

A comparison of the properties of WT luciferase and the 4TS mutant (both enzymes with C-His6) showed that the catalytic properties of the mutant

improved significantly compared to WT luciferase: the specific activity of the mutant increased by a factor of 2 and the K_m value for the ATP region decreased by a factor of 8. The kinetics of irreversible thermal inactivation of WT luciferase and the 4TS mutant was studied in the range of 37–55°C at pH 7.8 in a Tris-acetate buffer solution (close in composition to the buffer used in practice to determine ATP) (Fig. 13), as well as in a phosphate buffer (close in composition to the solutions used in a number of studies on luciferase mutagenesis). At 45°C, the thermal stability of the *L. mingrelica* 4TS luciferase mutant in a phosphate buffer increased 155-fold compared to WT luciferase. Over the entire temperature range studied, the 4TS mutant was significantly more stable than WT luciferase.

Thus, the 4TS mutant significantly exceeds both the wild-type enzyme and other known firefly luciferase mutants in terms of thermal stability and catalytic characteristics. As reagents for determining ATP and in vivo gene expression markers, luciferases are used in the temperature range from room temperature to 37°C. At 37°C, the 4TS mutant retains 70% of its activity after 2 days; i.e., its stability is sufficient for most practical purposes. The use of a highly efficient pET expression system and *L. mingrelica* thermostable firefly luciferase mutant allowed obtaining a luciferase producer (cloning vector pETL7, GenBank: HQ007050.1), which had a protein yield 3.5–4.0 times higher and a specific activity 4.4 times higher compared to luciferase obtained by the standard method based on the pLR plasmid. The use of metal chelate chromatography significantly reduced the enzyme purification time, and the luciferase preparation was obtained in a highly concentrated form [58]. Due to the high values of the activity yield and thermal stability of the resulting preparation, this luciferase producer was used for practical applications, including being used to produce an ATP reagent. The practical aspects of the use of the ATP reagent in bioluminescent ATP-metry are described in detail in a recently published monograph [59], which presents numerous examples of the use of this reagent, which is now commercialized. Bioluminescent ATP-metry is successfully used in sanitation, biomedicine, toxicology, solving environmental problems, the development and use of environmental technologies, antimicrobial drugs and food products, chemical-biological means of protection and anticorrosive agents, and new and effective biocatalysts and biotechnological processes [59].

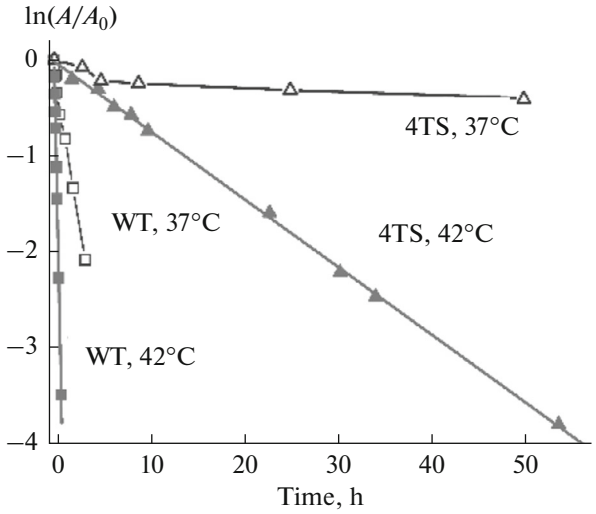

Fig. 13. Kinetic curves of thermal inactivation of the original (WT) and thermostable (4TS) *L. mingrelica* luciferase in tris-acetate buffer solution.

Emitter as an Intramolecular Label in the Active Site of Firefly Luciferase

The peculiarities of bioluminescence in the luciferin–luciferase system of fireflies are complex changes in the shape of the spectra and λ_{max} bioluminescence by variations in pH, temperature, and enzyme structure. As stated above and as confirmed by independent modern spectral studies [60], this is explained by the keto-enol tautomerism of the oxyluciferin molecule (Fig. 5). However, only one molecule of the electronically excited product is formed in the active center of the enzyme. Thus, the emitter molecule represents an intramolecular bioluminescent label that characterizes the properties of the emitter microenvironment at the moment of light emission [61, 62]. The superposition of two or three forms of the emitter, recorded in the bioluminescence spectra, indicates the coexistence in the reaction medium of different conformational forms of luciferase, which are in dynamic equilibrium:

$$E1 \leftrightarrow E2 \leftrightarrow E3.$$

Each of the luciferase conformers can contain only one of three forms of the emitter (ketone, enol, or enolate):

$$\underset{\text{Ketone}}{E1(LO{=}O)^*} \leftrightarrow \underset{\text{Enol}}{E2(LO{-}OH)^*} \leftrightarrow \underset{\text{Enolate}}{E3(LO{-}O^-)}.$$

The analysis of the bioluminescence spectra allowed us to identify qualitatively and quantitatively different enzyme conformers and changes in their concentration when varying the external conditions and the structure of luciferase. Monomodal bioluminescence spectra correspond to one conformer of the enzyme, which is determined by both the enzyme structure and pH or temperature values. In the case of not monomodal but more complex (bi- and even trimodal) bioluminescence, spectra are observed, which means that under these conditions in the reaction medium there are various enzyme conformers in which one or another form of the emitter is realized. Several examples were used to demonstrate how the analysis of bioluminescence spectra allowed us to draw conclusions about changes in the composition of

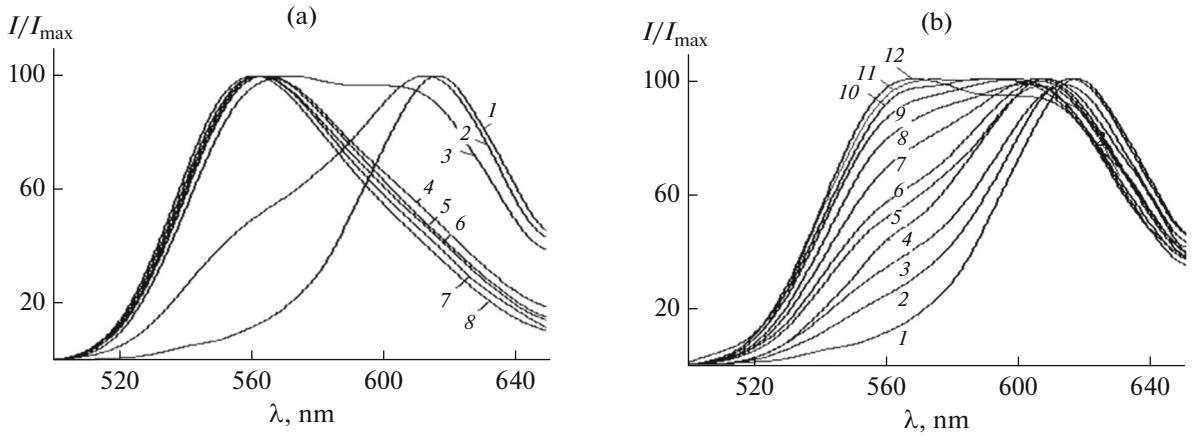

Fig. 14. Bioluminescence spectra for native (a) and mutant (b) firefly *L. mingrelica* luciferase at different pH values: (a) pH 5.6; 6.4; 6.8; 7.0; 7.6; 7.8; 8.0; 8.5 (*1–8*, respectively); (b) pH 5.6; 6.1; 6.4; 7.2; 7.6; 7.8; 8.0; 8.6; 8.9; 9.2; 9.6; 10.2 (*1–12*, respectively). The intensity at the maximum of each spectrum is normalized.

luciferase conformers due to changes in mutations or external conditions (pH and temperature).

The His433Tyr mutation in *L. mingrelica* luciferase led to a shift in $\lambda_{max.em.}$ bioluminescence from 566 to 606 nm at pH 7.8 (pH optimum activity) [50], which was explained by the change in the ratio of different forms of oxyluciferin (Fig. 14).

The use of the Gauss method for the decomposition of bioluminescence spectra allowed us to establish that the observed spectra are a superposition of the spectra of three forms of electronically excited oxyluciferin: enolate (LO–O^-, $\lambda_{max.em.}$ = 556 nm), enol (LO–OH, $\lambda_{max.em.}$ = 587 nm) and ketones (LO=O, $\lambda_{max.em.}$ = 618 nm). Changing the relative content of different forms of the emitter with varying pH led to a shift in the position of the maximum and a change in the shape of the bioluminescence spectrum. The relative abundance of each form of the enzyme–emitter complex was determined at different pH values. For WT luciferase, at pH ≥ 7.0, luciferase conformers containing the enol and enolate forms of the emitter were predominant, and conformers containing the ketone form of the emitter were predominant only at pH 5.6. The mutant luciferase at pH ≤ 6.1 was in the form of a conformer containing the ketone form of oxyluciferin. With increasing pH, conformers containing enol and enolate appeared, but their proportion become quite noticeable only at pH ~ 10.2.

It is known based on X-ray structural data for firefly luciferases, that the His433 residue is located in a flexible loop formed by residues Tyr427–Phe435, which connects the N- and C-domains of luciferase [45, 46]. This loop can be viewed as a "hinge," connecting two luciferase domains. The imidazole ring of the His433 residue forms a hydrogen bond with the carboxyl group of the Asp431 residue, which increases the rigidity of the hinge and reduces the amplitude of thermal fluctuations of the N- and C-domains relative to each other. This ensures a rigid fixation of the amino acid residues Thr529 and Lys531 from the C-domain, which are in the immediate vicinity of the thiazole group of oxyluciferin. When His433Tyr is replaced, hydrogen bonding becomes impossible, resulting in a decrease in the rigidity of the Tyr427–Phe435 hinge and an increase in the amplitude of thermal vibrations of the domains relative to each other. The emitter microenvironment becomes looser, which complicates keto-enol tautomerization. As a result, a shift in equilibrium towards the conformer containing the ketone form of the emitter is observed.

An increase in the temperature of the solution can also lead to an increase in the amplitude of thermal oscillations of domains relative to each other. At the same time, the microenvironment of the emitter also becomes "looser"; i.e., the concentration of the luciferase conformer that generates the red glow increases. This was shown especially clearly when analyzing the temperature dependencies of the bioluminescence spectra obtained at 10, 25, and 42°C for *L. mingrelica* luciferase and several of its single mutants at the Glu457 residue (Glu457Asp/Gln/Lys) [63]. For all luciferases, the total bioluminescence spectra were presented as superpositions of the spectra of the luciferase conformer with the green ($\lambda_{max.em.}$ = 554 ± 3 nm) and red ($\lambda_{max.em.}$ = 595 ± 5 nm) emitters. The WT-luciferase and the Glu457Asp mutant have similar temperature dependencies of bioluminescence spectra. For Glu457Gln mutants, the green conformer comprises only ~20% at 10°C and ~10% at 25°C. Luciferase with the Glu457Lys mutation has a monomodal spectrum with a maximum at 600 nm throughout the entire temperature range studied. At 42°C, for most mutants, the green glow disappears, since with increasing temperature the content of green luciferase conformers generating enolates becomes very low. At 42°C the proportion of the red emitter increases to

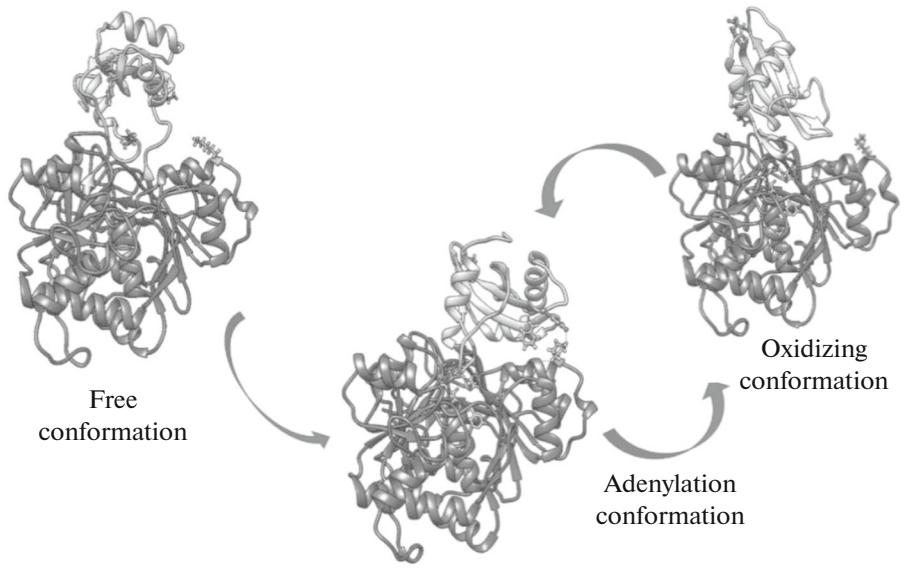

Fig. 15. Dynamic structure of firefly luciferase. Sequence of C-domain rotations during the catalytic reaction of *L. mingrelica* luciferase.

90% for WT luciferase, and to 100% for mutants. The analysis of the spectra shows that for all $\lambda_{max.em.}$ mutants the green and red components coincide with the values of $\lambda_{max.em.}$ for WT luciferase, and with a change in temperature only the ratio between their concentrations changes. Therefore, the structure of the emitter is independent of temperature, but the ratio between the different protein conformers changes with temperature. The loosening of the protein structure upon heating leads to a shift in the bioluminescence spectra towards the red conformer.

The Role of a Dynamic Protein Structure in the Catalysis of Firefly Luciferase

The ability of luciferase to catalyze three sequential reactions (Fig. 1) is determined by the special dynamic structure of the enzyme, due to which the N- and C-domains can take on different configurations relative to each other. At each stage, the structure of the active center of the enzyme is realized, which is most effective for catalyzing the given stage of the process. In 1999, a model was proposed [47], according to which, upon the binding of substrates, the free conformation is transformed into an adenylation conformation due to the rotation of the N- and C-domains by 90° relative to each other. This was later proved by the X-ray structural analysis of the luciferase complex with an analog of luciferyl adenylate [54], which is formed in the first stage of the enzymatic process. Then, under the influence of atmospheric oxygen, luciferyl adenylate is oxidized, forming electronically excited oxyluciferin. In the study of Branchini et al. [64], it was shown that during oxidation, an oxidizing conformation is realized in which both domains of luciferase are rotated relative to each other by 140°. According to the authors, this conformation is maintained at the moment of light emission [65]. To find out the conformation the enzyme globule actually has at the moment of light emission, we used the random mutagenesis method to search for such mutations of the C-domain that would specifically affect the bioluminescent properties of the system but would not disrupt the catalytic process and would not introduce noticeable changes in the enzyme structure. Such C-domain mutations have been found and their study has shed light on the role of the C-domain in color modulation and revealed new aspects of the domain alternation mechanism.

We showed that single Phe467Ser, Glu490Val, and Glu490Lys mutations in the C-domain of *L. mingrelica* luciferase had practically no effect on the stability, specific activity, and pH optimum of the enzyme activity, but sharply changed the pH sensitivity of the bioluminescence spectra [66]. Since the spectra reflect the emitter structure, we hypothesized that these residues influence the emitter microenvironment in the active site. Model structures of the enzyme were constructed in three known conformations (open, adenylated, and oxidative) (Fig. 15).

The structural analysis and experimental data have provided no evidence that these residues can influence the active site in the open or oxidative conformation. The localization of Phe467 and Glu490 residues relative to the N-domain changes dramatically during the reaction. In the free conformation, the side chains of these residues are localized on the surface of the C-domain, exposed to the solvent and do not participate in structure-forming interactions. In the adenylation conformation, they are in the interdomain space

and directly interact with the N-domain, which contains most of the active center groups. In the oxidative conformation, rotation of the C-domain brings these residues to the outer surface of the enzyme, moving them as far away from the N-domain as possible (Fig. 15). Thus, the oxidative conformation is necessary only for the initiation of the oxidation of luciferyl adenylate, and at the moment of light emission the enzyme returns into the adenylation conformation. Therefore, only in the adenylation conformation are the Phe467 and Glu490 residues involved in interactions with the N-domain and can alter the active site microenvironment during light emission, which occurs from the adenylation conformation of luciferase [66].

ABBREVIATIONS AND NOTATION

ATP	adenosine-5'-triphosphate
AMP	adenosine-5'monophosphate
LH_2	luciferin
LO	oxyluciferin

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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