Relationship between the structure of the protein globule and bioluminescence spectra of firefly luciferase

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Bioluminescence spectra for various native and mutant luciferases from fireflies and beetles were analyzed in the light of the known theoretical concepts on the influence of the microenvironment of the emitter on its emission spectra. The mechanism for the explanation of the nature of changing bioluminescence spectra for natural and artificial mutations of the amino acid residues in the protein globule of luciferases was proposed.

Key words: bioluminescence, luciferase, luciferin, ATP, fireflies, bioluminescence spectra, protein structure, mutation of amino acid residues.

Introduction

Beetle luciferase (luciferin 4-monooxygenase (ATP hydrolysis)*; luciferin: oxygen 4-oxidoreductase (decarboxylation, ATP hydrolysis), EC 1.13.12.7) catalyzes the oxidation of firefly luciferin with air oxygen in the presence of MgATP.[1] The reaction is accompanied by the visible light emission with a quantum yield of ~90%.[2] As shown in Scheme 1, at the first stage the enzyme binds the substrates, luciferin **1** and ATP. In the ternary complex luciferin covalently interacts with ATP to form the mixed anhydride of carboxylic and phosphoric acids: luciferyl-adenylate (**2**) and pyrophosphate. Luciferyl-adenylate (**2**) is oxidized by air oxygen through several intermediate stages to produce cyclic peroxide, dioxetanone **3**. The transformation of dioxetanone results in the formation of the biradical, whose decarboxylation affords the reaction product, oxyluciferin (**4**), in the singlet electron-excited state. The keto form of oxyluciferin (**4a**) is rapidly transformed into the enol form (**4b**) when the H(5) proton is removed (Scheme 1). Then electron-excited oxyluciferin is deactivated with the emission of a photon. The emitter structure in the bioluminescence system of fireflies was revealed by the study of the fluorescence properties of oxyluciferin in model systems.[3] At pH >7.0 oxyluciferin is characterized by the yellow-green fluorescence (λ_{max} = 560 nm), and when the pH decreases the red fluorescence appears (λ_{max} = 612 nm).

Luciferases were isolated from several species of beetles, such as fireflies (*Lampyridae* family), click-beetles (*Elateridae* family), and rail-road worms (*Phenogodidae* family). The scheme of the chemical reaction catalyzed by these enzymes and the emitter structure are identical for all isolated luciferases of insects. The main difference is the color of the bioluminescence. In nature (*in vivo*) fireflies emit the yellow-green light (540—580 nm),[4—7] click beetles emit in the range from green to orange,[5,8] and rail-road worms emit in the range from green to red (536—638 nm).[7,9] When the reaction is performed *in vitro*, the observed color of bioluminescence coincides with the color *in vivo* at neutral pH but shifts to the red region if the reaction medium is acidified.[6] Thus, the main factors determining the color of bioluminescence are the properties of the microenvironment of the emitter localized in the active site of the enzyme.

Presently, the primary structures of 17 luciferases of fireflies and beetles are known. They were isolated from various species collected in the USA, Russia, and South America (Table 1). All luciferases consist of one protein chain (542—552 residues), contain no cofactor, and have a similar amino acid composition. More than a half of amino acids are nonpolar or ambivalent. The number of charged residues is also virtually the same, and the main distinction is in the number of Trp and Cys residues (Table 1). The homologies of amino acid sequences for various luciferases correspond to the ratios based on their biological classification. For luciferases of the *Luciola* family, the homology is about 80%, and beginning from the 200th residue, it is higher than 90%. Luciferase from *L. mingrelica* is most close in structure to luciferase of Japanese fireflies *Hotaria parvula* (98% of homology). *Luciola* luciferases have a lower homology with *Photinus pyralis* luciferase (67%) and still lower with luciferases of click-beetles (43%). At the same time, the homology for luciferases isolated from Jamaican and Brazilian beetles reaches 80%.[10]

* ATP is adenosine-5′-triphosphate.

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Scheme 1

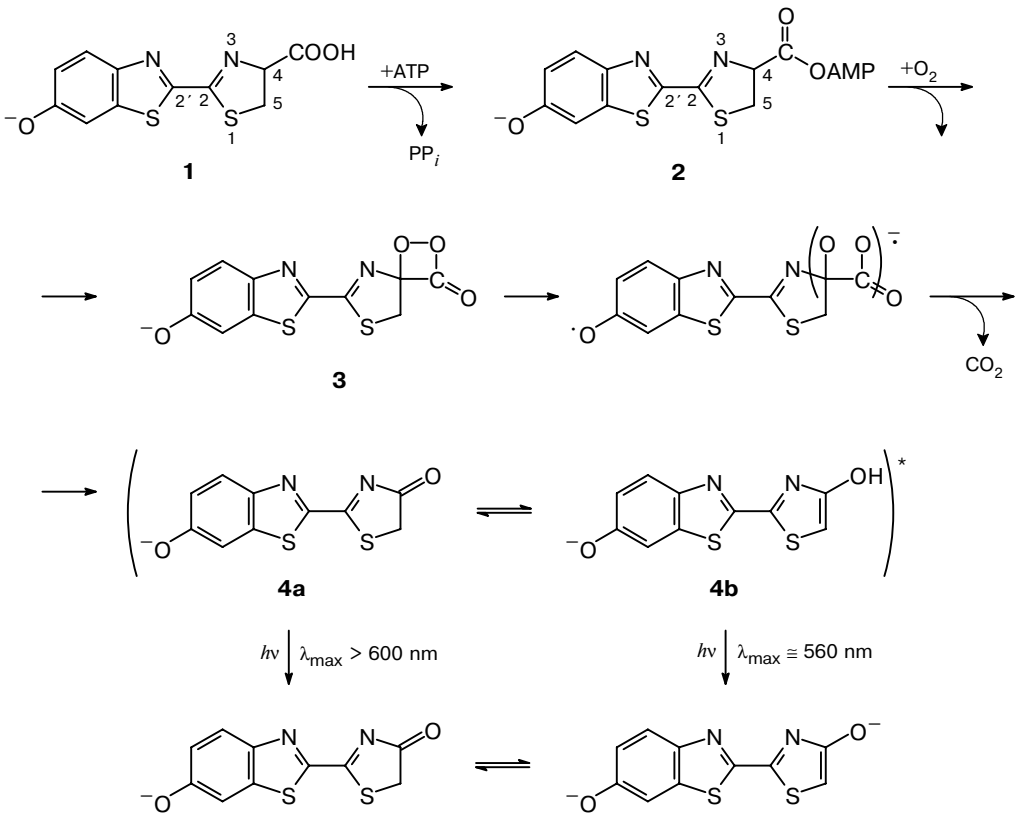

Table 1. Cloned luciferases from fireflies and beetles

Year of cloning	Luciferases	Number of amino acid residues	λ_{max}* /nm	p*I***	Trp	Cys	References
1987	*Photinus pyralis*	550	562	6.42	2	4	33
1989	*Pyrophorus plagiophtalamus*						18
	Green LucGR	543	546	6.69	3	13	18
	Yellow green LucYG	543	560	6.39	2	13	18
	Yellow LucYE	543	578	6.69	2	13	18
	Orange LucOR	542	593	6.69	2	13	18
1989	*Luciola cruciata*	548	562	7.07	1	8	34
1992	*Luciola lateralis*	548	552	6.5	1	7	35
1993	*Luciola mingrelica*	548	570	6.24	1	8	36
1994	*Photuris pennsylvanica*	552	560	7.55	1	11	37
1994	*Photuris pennsylvanica*	545	538	8.39	2	7	37
1995	*Hotaria parvula*	548	568	6.27	1	8	38
1995	*Pyrocoelia miyako*	548	550	6.12	2	9	38
1995	*Lampyris nocticula*	547	550	6.09	1	9	39
1999	*Rhagophthalmus ohbai*	543	554	***	***	***	40
1999	*Phrixothrix viviani*	545	549	6.26	1	7	41
1999	*Phrixothrix hirtus*	546	622	7.0	1	9	41
1999	*Pyrearinus termitilluminans*	543	538	6.75	2	10	10

* The maxima of the bioluminescence spectra for the luciferin-luciferase system are presented.
** p*I* is isoelectric point, *i.e.*, pH at which the net charge of a protein molecule is zero.
*** The corresponding data are not presented in Ref. 40.

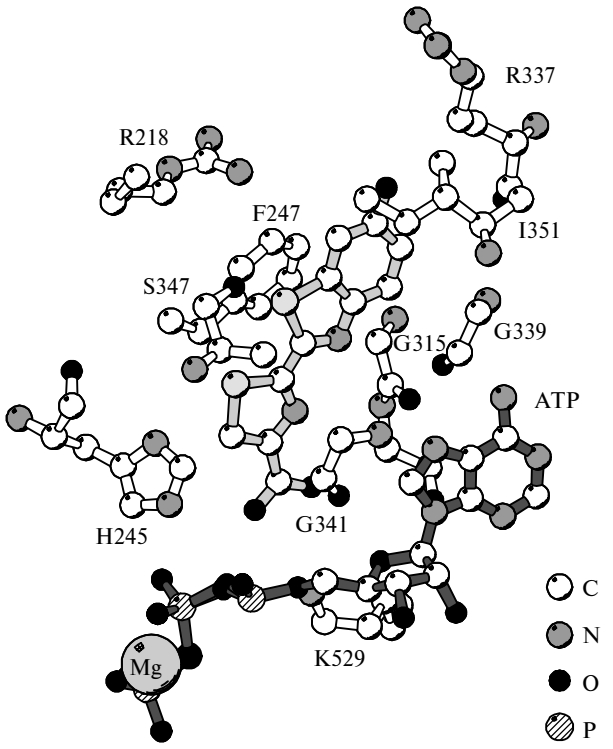

Fig. 1. Model of the structure of the active site of firefly *Photinus pyralis* luciferase.[13]

Based on data on the tertiary structures of *Photinus pyralis* luciferase[11] and the enzyme-substrate complex for tyrocidine-synthetase resembling that of luciferase in spatial structure and ability to adenylate an organic acid, the authors[12,13] proposed the models of the luciferase-luciferin-ATP complex structure (Fig. 1) and revealed the conservative amino acid residues, which presumably form the binding site of luciferase substrates and, correspondingly, the microenvironment of the reaction product, oxyluciferin. However, despite obvious success in studying luciferases, the molecular basis for distinctions in bioluminescence spectra for native and mutant forms of luciferases from various sources remain intriguing.

In this review, available experimental data on bioluminescence spectra of various native and mutant luciferases are analyzed in the light of the known theoretical concepts on the medium influence on emission spectra and the mechanism that explains the nature of changing the bioluminescence color is proposed. The elucidation of a relationship between the structure of the luciferase protein and the maximum in the bioluminescence spectrum is of both theoretical and practical interests because luciferases and their genes are widely used presently as markers in studying various biochemical processes under *in vitro* and *in vivo* conditions.[14] The use of luciferases generating the light in various regions of the visible spectrum provides additional analytical potentialities for bioluminescence microanalysis.[15]

Theoretical concepts on the influence of the medium on emission spectra

The influence of the microenvironment on emission spectra is diverse, and no general theory explaining the observed phenomena has been developed in present time. According to the accepted photophysical views on the medium influence on spectra, the effects observed can be divided into general (nonspecific) and specific.[16] It is difficult or even impossible to distinguish effects of two type in real situation but this approach facilitates revealing the prevailing mechanism.

Considering the general effects of the medium on emission spectra, we can imagine the emitter as a dipole in the continuous dielectric medium. The interaction between solvent and emitter molecules influences on the difference of the energies between the ground and excited states and, correspondingly, on the shift of the luminescence maximum. On the other hand, the interaction energy depends on the dynamic characteristics of the interaction, *i.e.*, the rate of reorientation of the solvent molecules around the excited emitter molecule, rather than the structures of the solvent and emitter. When the molecule transits to the electron-excited state, its electronic structure is instantly (within 10^{-15} s) rearranged. Thus, in the "initial moment," the emitter is in the nonequilibrium state with respect to the surrounding medium (Fig. 2). The difference between the energies of the excited and ground states in this moment is maximum and, correspondingly, the wavelength corresponding to the luminescence maximum is minimum. During the reorientation of the solvent molecules and approaching the "equilibrium" state, the energy difference decreases, which results in the shift of the luminescence maximum to the long-wave region. If the excited molecules had no time to be reoriented within the lifetime of the molecule, the difference between the energies of the excited and ground states is minimum, as has already been mentioned, and the luminescence, by contrast, is shortest-wave. And *vice versa*, in the case where the solvent molecules have time to be reoriented and the equilibrium state appears between the medium and emitter, the energy of the latter decreases by the value of the solvation energy, and the luminescence spectrum is shifted to the long-wave region. In the intermediate situation, the maximum of the emission spectrum lies between the ultimate positions indicated. The property of the solvent characterizing the mobility and rate of reorientation of molecules is named the orientational polarizability (Δf). The higher the orientational polarizability, the faster and easier the reorientation of molecules of the given solvent as a response to the introduction of the excited dipole into the medium. The orientational polarizability depends, in turn, on the dielectric constant (ε) and refractive index (n) of the medium according to Eq. (1).

$$\Delta f = (\varepsilon - 1)/(2\varepsilon + 1) - (n^2 - 1)/(2n^2 + 1). \qquad (1)$$

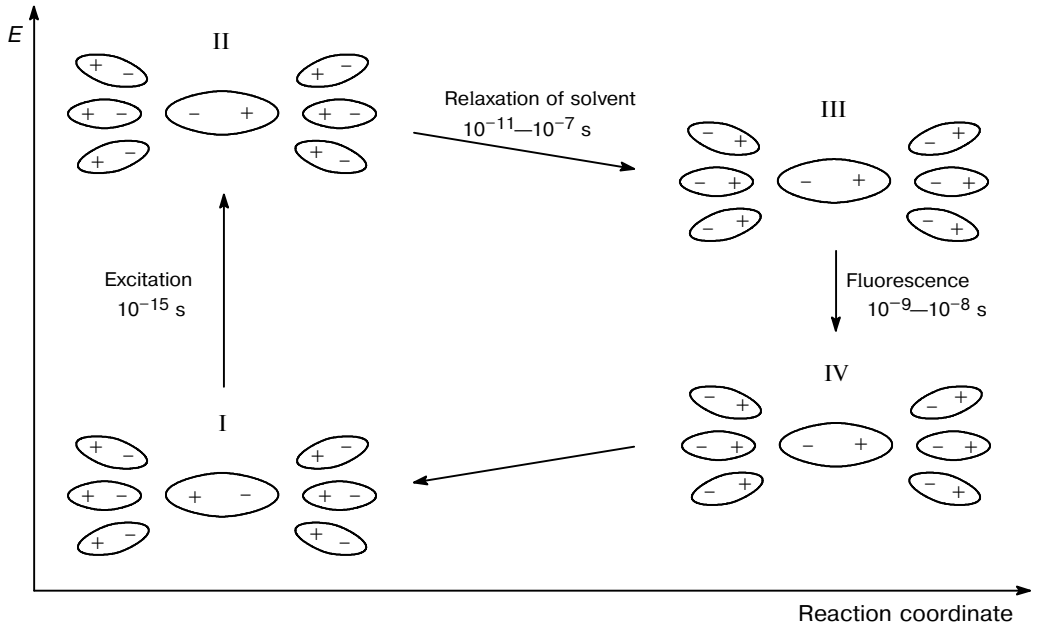

Fig. 2. Diagram of the excitation and emission energy levels (E) of the molecule.

The dependence of the Stokes shift ($\Delta\nu$) (the difference in energies between the excited and ground states) on the orientational polarizability of the solvent (Δf) is described by the Lippert equation (Eq. (2)).

$$\Delta\nu = A\Delta f(\mu^* - \mu)^2 + B, \qquad (2)$$

where μ^* and μ are the dipole moments of the molecule in the excited and ground states, respectively; and A and B are constants. Thus, the maximum changes in the emission spectrum are observed for emitters for which the maximum change in the dipole moment is observed on going from the ground to excited states and which exist in the solvent with the highest orientational polarizability. The shape of the emission spectrum remains virtually unchanged when the maximum is shifted to the long-wave region with an increase in the polarizability of the solvent used. As an example, we can present the dependence of the fluorescence spectra of firefly luciferin on the orientational polarizability in different solvents (Fig. 3).[17]

The nature of the specific influence of the solvent on the emission spectra is in the formation of hydrogen and acid-base bonds or the charge transfer complexes between the functional groups of the emitter and substances in its microenvironment. In this case, additives of trace amounts of substances, which do not virtually change the volume properties of the solvent, change considerably the emission spectra. The specific interactions of the emitter with the solvent can occur in both the ground and excited states. When the interaction occurs only in the excited state, it does not affect the absorption spectra. The specific interactions are characterized by the appearance of new bands in the spectrum. The dependence of the fluorescence spectra of firefly

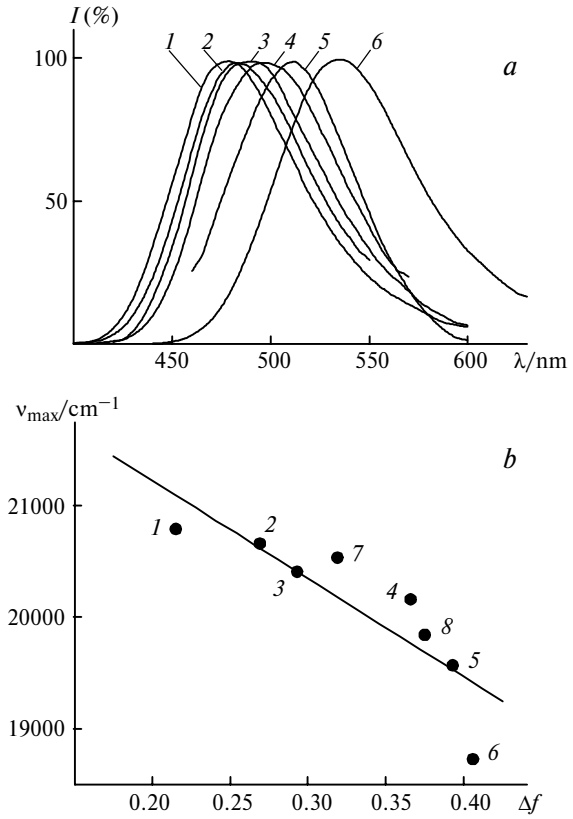

Fig. 3. Fluorescence spectra of luciferin in various solvents (*a*) and the dependence of the fluorescence maximum of luciferin on the polarizability of the solvent (*b*): *1*, diethyl ether; *2*, butyl acetate; *3*, ethyl acetate; *4*, ethyl methyl ketone; *5*, acetonitrile; *6*, aqueous buffer solution; *7*, dichloromethane, and *8*, acetone.[17] The polarizability of the solvent (Δf) was calculated by equation (1).

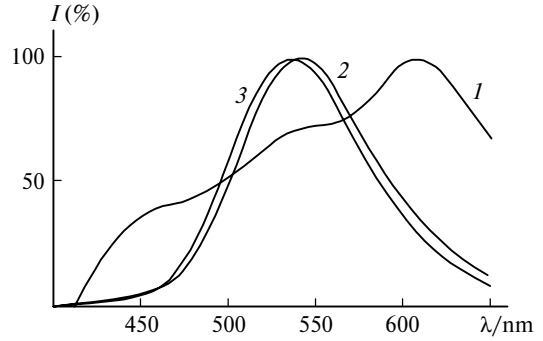

Fig. 4. Fluorescence spectra of luciferin at different pH[3]: *1*, 1.03; *2*, 2.76; and *3*, 7.16.

luciferin on the pH (Fig. 4) is an example of the specific influence of the solvent on the emission spectra of the emitter.[3] When the pH of the medium decreases, both the absorption and fluorescence spectra exhibit the appearance of new bands, which indicate the appearance of the new form of the luciferin molecule with changing the pH of the medium because a decrease in the pH of the medium results in the protonation of the carboxy and phenolate groups of the molecule.

The presented above theoretical consideration of the factors and mechanisms of the influence of the medium on the emission spectra suggest that the shape of the fluorescence spectra of the emitter in various media allows one to judge what mechanism of the solvent influence prevails: general or specific. In the case of the general mechanism, the observed shift of the spectrum is relatively small, the shape of the spectrum do not substantially change, and the direction of the shift of the spectrum depends on the change in the orientational polarizability of the medium. In the case of the specific influence, a substantial shift of the emission spectrum accompanied by the appearance of new spectral elements is observed.

Bioluminescence spectra of various luciferases and their mutants

It has been found that the large class of Jamaica click beetles exhibit luminescence with different bioluminescence maxima.[4] Further studies showed that cDNA* isolated from ventral organs of these beetles and expressed in *Escherichia coli* coded four different luciferases with 94—99% homology of sequences and bioluminescence maxima from green to orange (546, 560, 578, and 593 nm, respectively).[18] The shape of the spectra is the same and, hence, they cannot be considered as a result of the superposition of the spectra of the ketonic and enolic forms of oxyluciferin (red and yellow-green emitters).[19] The hybrid proteins were constructed by a combination of four parts of the sequences. As a result, the set of 31 proteins, including native, was obtained. The authors[20] analyzed the relationship between the amino acid replacements and bioluminescence spectra in this set of luciferases, assuming the additivity and independent character of the influence of replacements. Two sets of amino acids were identified (Arg223Glu, Leu238Val) and (Ser247Gly, Asp352Val, Ser358Thr), which result in the red shift of the bioluminescence maximum.

A series of random mutants differed by the maximum in the bioluminescence spectrum was obtained for *Luciola cruciata* luciferase (λ_{max} = 562 nm).[21] It was shown that point mutations of Ser286Asn, Gly326Ser, His433Tyr, and Pro452Ser resulted in the shift of the bioluminescence spectrum to the red region. At pH 7.8, λ_{max} for the mutants lies at 607, 609, 612, and 595 nm, respectively. The substitution of Val239Ile resulted in the shift of the bioluminescence maximum to the green region (λ_{max} = 558 nm). The position of the maximum in the bioluminescence spectrum for the mutants remained unchanged with a decrease in the pH to 6.0.

For luciferases from fireflies *Pyrocoelia miyako* (λ_{max} = 550 nm) and *Hotaria parvula* (λ_{max} = 568 nm), 5 chimeric mutants were obtained containing different fragments of molecules of these two luciferases.[22] Analysis of the bioluminescence spectra of the mutants showed that this is precisely the protein fragment localized between Val209 and Ala318 which is responsible for the observed difference in bioluminescence maximum for two luciferases studied.

A series of point mutants in which the Ser286 residue was substituted by other amino acid residues was obtained for *L. mingrelica* luciferase.[23] The mutation data resulted in the shift of the bioluminescence maximum from 570 nm (for the native enzyme) to 608, 609, 613, and 619 nm in the series of mutants of Ser286Lys, Gln, Tyr, and Leu. In addition, *Hotaria parvula* luciferase with the point mutation of His433Tyr, whose bioluminescence maximum is also shifted to the red region (λ_{max} = 610 nm), was obtained by the method of random mutagenesis.[24] It should be emphasized that, in all cases described above, authors observed the shift of the maximum in the bioluminescence spectrum without a substantial change in the shape of the spectra.

Among mutants of *Photinus pyralis* luciferase, the mutants by His245 and Thr343 had the bioluminescence spectra different from those for the native enzyme (Table 2).[25] Mutations of His245Arg, Phe, Ala, Gln, and Asn resulted in a considerable broadening and changing the shape of the bioluminescence spectra. The bioluminescence maximum at pH 7.8 shifted from 579 to 613 nm in the series of mutations indicated above. The resulting spectra were the sum of the spectra of two forms of the emitter, ketone and enol, and were pH-dependent. It is most likely that the mutations of His245 affect the pK value of the keto-enol tautomerism thus shifting the equilibrium between the tautomers to the region of higher pH. Only the His245Asn mutant exhibited only the red bioluminescence characteristic of

* cDNA is the complementary DNA.

Table 2. Specific activity and spectral characteristics of bioluminescence for mutants of *P. pyralis* luciferase[25]

Enzyme	I_{rel} (%)	λ_{max}/nm		$\lambda_{558}/\lambda_{613}$ pH 7.8
		pH 7.8	pH 5.5	
Native	100.0	558	613	3.1
Mutants				
His245Phe	18.4	595	620	0.6
His245Ala	26.4	604	620	0.5
His245Arg	0.05	579	618	0.9
His245Gln	1.6	606	620	0.4
His245Asn	24.8	613	617	0.3
His245Asp	0.23	617	618	0.15
Thr343Ser	26.4	560	617	2.7
Thr343Ala	1.1	617	621	0.1

Note. I_{rel} is the relative activity; λ_{max} is the bioluminescence maximum; $\lambda_{558}/\lambda_{613}$ is the ratio of bioluminescence intensities at 558 and 613 nm.

ketone. The Thr343Ser and Thr343Ala mutants catalyzed the yellow-green and red emissions, respectively. The bioluminescence spectra were identical to those for native *P. pyralis* luciferase at pH 7.8 and 5.5, respectively.[25]

Chimeric luciferases were prepared by the fusion of the regions of sequences 1—344 and 345—545 of two enzymes isolated from Brazil beetles *Phrixothrix viviani* (λ_{max} = 548 nm) and *Phrixothrix hirtus* (λ_{max} = 623 nm).[26] The authors showed that the color of bioluminescence of the chimeric protein depends only on the structure of region 1—344. The point mutation of Arg215Ser in *Phrixothrix viviani* luciferase, which generates the green luminescence, resulted in the shift of the bioluminescence maximum by 40 nm to the red region and in a considerable decrease in the activity. For *Phrixothrix hirtus* luciferase, which generates the red luminescence, the same mutation had a weak effect on the bioluminescence spectrum.

Mechanisms of changing the bioluminescence color for the luciferin-luciferase system proposed in literature

Many of the results mentioned above had been obtained before the tertiary structure of luciferases and the model structure of their active site became known. It was difficult to reveal the structural factors, which determine the changes observed in the bioluminescence spectra, knowing only the primary structure. Nevertheless, several probable mechanisms for the explanation of variations of the bioluminescence color in native and mutant luciferases of beetles and fireflies were proposed in literature.

The authors[27] proposed that the shifts in the bioluminescence spectrum can be explained by a change in the polarity of the oxyluciferin-binding site in luciferase. This mechanism is similar to the dependence of the spectral properties of the dissolute on the solvent polarity. However, this mechanism cannot explain the existence of two forms of oxyluciferin (ketonic and enolic) at different pH.

According to another mechanism, the changes in the bioluminescence spectra, especially with changing the pH of the solution, are induced by the keto-enol tautomerism of oxyluciferin. It is assumed that the protein environment of the emitter contains an amino acid, whose lateral residue acts as a base removing the H(5) proton to result in the yellow-green bioluminescence. In an acidic medium (pH < 6.0), the protein structure changes near the emitter, so that this base either is protonated or changes its position and, hence, cannot perform its function. As a result, the red luminescence arises. At intermediate pH values, the bioluminescence spectrum consists of two components: ketone and enol. This mechanism explains the changes in the shape of the bioluminescence spectra and the ratios of maxima of two forms (ketone ad enol) with changing the pH of the reaction medium. This is characteristic of many native luciferases, especially *in vitro* and for mutants by His245, but cannot explain the shifts of the bioluminescence maxima without changes in the shape of the spectra, which were observed for firefly luciferase mutants and for native click-beetle luciferases.

The authors[28] proposed mechanism based on the assumption that the luciferase reaction afforded oxyluciferin in the form of ketone, which can exist in two stereoforms. These forms appear when the benzothiazole and thiazole cycles rotate about the C(2)—C(2′) bond (see Scheme 1). The authors′[28] quantum-chemical calculations showed that the stereoform with the highest energy of excited luciferin was not planar and the planes of two cycles were turned relatively to each other by 90°. This unexpected result was in a consequence of the intramolecular charge transfer in such a "twisted" conformation. Perhaps, red luminescence appears when the free rotation about the C(2)—C(2′) bond is realized in the keto form of excited oxyluciferin. In the case of steric hindrances for this rotation, oxyluciferin exists in the high-energy state, whose deactivation results in the green color emission. It is assumed that in the active site of luciferase oxyluciferin has such a rigid conformation. However, the partial denaturation of luciferase (for example, with decreasing the pH) results in the free rotation of oxyluciferin, which is transformed in the low-energy rotational conformation, whose deactivation is accompanied by red luminescence. Amino acid replacements can also result in various degrees of rotational freedom of the emitter and thus induce smooth changes in the bioluminescence color. However, this mechanism cannot explain the green fluorescence of the enolic forms of oxyluciferin and its analogs. On the other hand, the quantum-mechanical calculations showed[29] that the planar conformation was optimum for excited oxyluciferin. The calculation of the structure of the excited luciferin molecule in a dimethyl sulfoxide

solution resulted in a similar conclusion.[30] In addition, excitation increases sharply the dipole moment of the oxyluciferin molecule and even changes its direction.[29] As it is known, the higher the difference between the dipole moments of the emitter in the ground and excited states, the greater the observed shift of the maximum in the luminescence spectrum. In our opinion, the mechanism proposed by McCapra[28] hardly takes place in practice because it is not confirmed by either quantum-mechanical calculations of the structures of luciferin and oxyluciferin molecules or experimental data.

The effects of various physicochemical characteristics of amino acid residues, which are varied in the structure of click-beetle luciferases on the maxima in their bioluminescence spectra were compared.[31] The shape of the spectra for different luciferases was shown to remain unchanged, *i.e.*, the energy of the excited state of enol changes, and the ratio of the ketone and enol concentrations remains unchanged. The best correlation is observed between the position of the maximum in the bioluminescence spectra and the orientational polarizability of the amino acid residues in the region of the amino acid sequence between residues 223 and 352.[31]

General and specific influence of the microenvironment on bioluminescence spectra for native and mutant luciferases

Analyzing bioluminescence spectra different in the shape and maximum for native and mutant luciferases, most authors tended to find one or several "key" amino acid residues in molecules of luciferases responsible for the shifts observed in bioluminescence spectra. However, as it is shown above, similar shifts in the spectra were observed for mutations (natural or artificial) of amino acid residues arranged in different regions of the sequence. For example, the red shift was observed for natural mutations of Arg223Glu, Leu238Val, Ser247Gly, Asp352Val, and Ser358Thr in click-beetle luciferases[19] and for artificial mutations of both Ser286Asn and His433Tyr for highly homological *L. cruciata*[21] and *L. mingrelica* luciferases.[23] It follows from this that single "key" amino acid residues responsible for shifts in bioluminescence spectra barely exist. Similar changes in the spectra can be induced by mutation of one or several amino acid residues if this changes similarly the physicochemical characteristics of the microenvironment of the emitter. This is precisely the approach which agrees with the described above theoretical concepts about the general (nonspecific) and specific effects of the microenvironment on the position of the bioluminescence maximum.

Let us consider how the protein environment can affect the luminescence spectrum of excited oxyluciferin formed in the active site of enzyme. If excited oxyluciferin exists in the strongly nonpolar microenvironment, it is incapable of interacting efficiently with this environment. Then the difference in the energies of the excited and ground states of oxyluciferin is maximum and the emission is the shortest-wave, *i.e.*, in the green-blue spectral region. The same result is given from the protein microenvironment with a low polarizability of amino acid residues and a high rigidity of the protein fragment, although such a microenvironment can efficiently interact with the emitter. In this case, amino acid residues in the microenvironment of oxyluciferin had no time to be reoriented within the lifetime of the excited state of oxyluciferin, and the emission also occurs from the higher energy level. If the polarizability and mobility of the protein fragment surrounding the excited oxyluciferin are high, the maximum in the bioluminescence spectrum is shifted to the long-wave, orange-red spectral region. In this case, the amino acid residues in the microenvironment of oxyluciferin had time to be rearranged, and the energy of the emitter decreases. The stronger the interaction of luciferin with the protein and the higher the mobility of the protein fragment in the active site of enzyme, the longer-wave the luminescence. This general (nonspecific) influence of the polarizability of the microenvironment on the bioluminescence spectra is observed, in our opinion, in the cases where mutations touch the amino acid residues, which are located out of the active site but affect its polarizability. In these cases, as a rule, a good correlation between the polarizability of the introduced residue and the value of the bioluminescence maximum is observed.

The nonspecific mechanism of the shift of the bioluminescence maximum is illustrated well by the data for four beetle luciferases.[19] The linear dependence between ν_{max} of bioluminescence and the total polarizability of the amino acid residues in regions 223—224 and 247 (main contribution) and 352—358 (minor contribution) was shown[32] for luciferases of click beetles. The longer-wave maximum of bioluminescence corresponds to the enzyme with a higher polarizability of region 223—224.

The results on mutagenesis of firefly luciferase showed that a similar shift of the bioluminescence maximum (without changing the shape of the spectrum) is observed in fact when mutations do not touch directly the amino acid residues of the active site. Analysis of the tertiary structure of *L. mingrelica* luciferase shows that the highly conservative residue Ser286 is localized rather far from the active site[13] (Fig. 5). For both *L. mingrelica*[23] and *L. cruciata* luciferases[21] mutations in the Ser286 residue resulted in the red shift of the bioluminescence maximum and correlated with an increase in the polarizability of the residue introduced by mutation (Table 3). Of course, as it should be expected, some structural changes also occur in the enzyme. They can be explained by the proposed model of the active site[13] (see Fig. 1). As Fig. 6 shows for *L. mingrelica* luciferase, the Ser286 residue (Ser284 in *P. pyralis* luciferase) is linked by the chain of hydrogen bonds to Arg337, which is localized in the immediate vicinity from the active

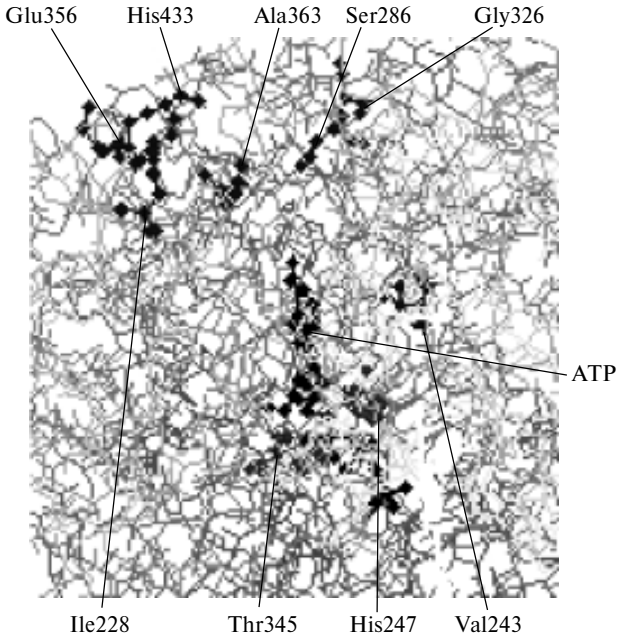

Fig. 5. Structure of the region of the *Luciola mingrelica* luciferase molecule where the mutated residues are designated.

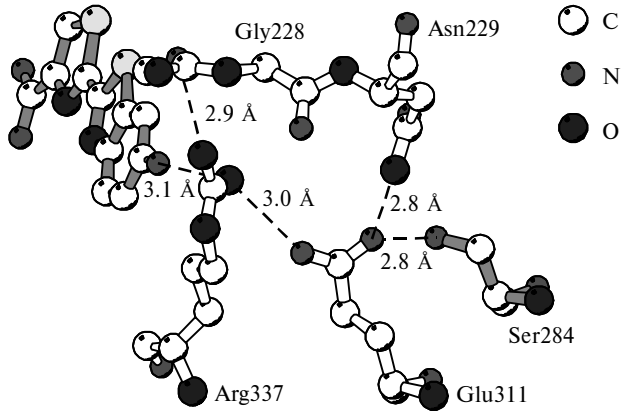

Fig. 6. Chain of hydrogen bonds between Ser284 (Ser286 in *Luciola mingrelica* luciferase) and luciferin for *Photinus pyralis* luciferase.[13]

site.[13] All residues bound by these hydrogen bonds are highly conservative, which implies the significance of this system of hydrogen bonds for maintaining the correct conformation of the active site. The disruption of the hydrogen bond between the Ser residue and adjacent groups and replacement of the residue with a small volume by more bulky residues change the conformation in the β-layers of 286—289, 313—315 and 338—340, due to which the mobility in the region of the active site and its polarizability may increase.

The mutation of His433Tyr in *L. cruciata* and *Hotaria parvula* luciferases, which results in the shift of the bioluminescence maximum from 570 to 610 nm, has, most likely, a similar indirect effect on the polarizability

Table 3. Relationship between the polarizability of the residue in position 286 and the maximum in the bioluminescence spectrum for native and mutant *L. mingrelica* luciferases

Residue	Polarizability[42] *	λ_{max}[23]/nm
Ser	3.26	580
Asn	5.5	607
Gln	7.2	609
Lys	9.38	608
Tyr	12.9	613
Leu	8.0	619

* The polarizability values were calculated by Eq. (1).

of the active site.[24,21] The polarizability of residue 433 increases from 8.86 (for His) to 12.9 (for Tyr). In addition, residue His433 is localized on the surface of the protein globule of luciferase. It can bear a positive charge, whereas the Tyr residue, which replaces it, has no charge, due to which some charge reorganization of lateral chains of the surrounding residues is possible. The charged residues Asp431, Glu432, Asp359, Glu356, and Lys366 are located within the interaction radius of the His433 residue. The removal of the positive charge from His433 can destabilize this cluster, and the negatively charged lateral chains will tend to move away from each other. The binding site of the adenine cycle of ATP is located at a distance of about 11.5 Å from the lateral chain of His433 and, perhaps, the substitution of His433Tyr will result in some reorganization of in the active site of luciferase, will increase the mobility of the groups of the active site and its polarizability.

The specific mechanism of the influence of the microenvironment on bioluminescence spectra is manifested when either the external pH is varied or for mutations of the active site groups involved in keto-enol tautomerism. Only the spatial structure of *Photinus pyralis* luciferase has been solved to date by X-ray diffraction analysis. Computer simulation showed that the tertiary structure of *L. mingrelica* luciferase (homology with *Photinus pyralis* luciferase about 67%) is virtually the same as that of *Photinus pyralis* luciferase.[13] The computer models of the active sites of both luciferases are also identical by the amino acid composition[12,13] (see Fig. 1), although the enzymes somewhat differ by maxima in the bioluminescence spectra (560 and 570 nm for *Photinus pyralis* and *L. mingrelica* luciferases, respectively).

Mutations in the active site of luciferase can induce a change in the pK of keto-enol tautomerism and, consequently, a change in the ratio between the concentrations of ketone and enol. When this ratio increases, the red component becomes pronounced in the bioluminescence spectrum. A similar mechanism takes place, most likely, for His245 and Thr343 mutants of *P. pyralis* luciferase (see Table 2).[25] These residues are absolutely conservative in all luciferases. According to the model proposed for the active site of luciferase,

imidazole of His245 is located at the inlet of the luciferin-binding pocket near the carboxyl group of luciferin and the γ-phosphate group of ATP (see Fig. 1). Mutations of His245Arg, Phe, Ala, Gln, and Asn result in a considerable broadening of the bioluminescence spectra. The bioluminescence maximum at pH 7.8 is shifted from 579 to 613 nm in the indicated series of mutations. The spectra are the sum of the spectra of two forms of the emitter, ketone and enol, and depend on the pH. The His245Asn mutant exhibits only the red bioluminescence characteristic of ketone. In this case, the shifts in the spectra do not correlate with the polarizability of the amino acid residue introduced by mutation. This proves the specific mechanism of the influence of mutations of the His245 residue on the bioluminescence spectra. In the model proposed for the active site of luciferase, the localization of His245 is that this residue can be precisely the base which participates in the keto-enol tautomerism. However, this is likely highly impossible because the emission spectra for mutants of His245Arg and Phe, which have no nucleophilic functional group, contain a significant fraction of the green light. Green emission can be favored by the positive charge on His245 and lateral nonpolar groups. A considerable fraction of the green luminescence for the His245Phe mutant containing the large aromatic residue can be a consequence of steric hindrances, due to which the enol conformation of the emitter is detected. The differences observed in the bioluminescence spectra for His245 mutants cannot be explained by any one property of groups introduced instead of His. Here we see the direct influence of both general and specific effects of the microenvironment on bioluminescence spectra.

For the Thr343Ser mutant of *P. pyralis* luciferase, the bioluminescence spectra are identical to those for native luciferase, whereas the Thr343Ala mutant exhibits the strong shift of the spectrum to the red region. The lateral OH group in position 343 is necessary for the normal yellow-green luminescence of *P. pyralis* luciferase.[25] This group can participate in the elimination of the H(5) proton of the luciferin molecule during its enzymatic oxidation. The absence of the OH group in the Thr343Ala mutant impedes the elimination of the H(5) proton, due to which the activity of the mutant enzyme decreases by almost 99%.

As can be seen, mutations in the active site of the enzyme result in changes in the bioluminescence spectra and also affect substantially the catalytic properties of the enzyme (see Table 2), as a rule, decreasing considerably the catalytic activity of luciferase, increasing K_m, *etc.* At the same time, natural mutants of luciferases with different bioluminescence spectra retain a high catalytic activity. This can be a consequence of the fact that these mutations do not touch the groups of the active site but affect its physicochemical properties by the nonspecific mechanism considered above.

The structure of a part of the *Luciola mingrelica* luciferase molecule in a complex with ATP is shown in Fig. 5. Two amino acid residues Thr345 and His247, which belong to the active site, are located near ATP. They correspond to the Thr343 and His245 residues in *Photinus pyralis* luciferase. The His433, Gly326, and Ser286, residues, whose mutations in luciferases from the *Luciola* genus result in the shift of the bioluminescence maximum to the red region, are remote from the active site. However, an increase in their polarizability during mutation has a noticeable effect on the bioluminescence spectra. The Ile228, Val243, Gly326, Clu356, and Ala363 residues correspond to the Arg(Glu)223, Leu(Val)238, Asp(Val)352, and Ser(Thr)358 residues in click-beetle luciferases, whose natural mutations (indicated in parentheses) induce the shifts of the bioluminescence maximum from 546 to 593 nm. These residues are also located outside the active site of luciferases. This additionally favors the described relationship between the structure of the protein globule of luciferases and their bioluminescence spectra.

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