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# Interaction of Dimethyl- and Monomethyloxyluciferin with Recombinant Wild-Type and Mutant Firefly Luciferases

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**Abstract**—Dissociation constants ($K_s$) in the pH range 6.5-9.0 for complexes of luciferin, dimethyloxyluciferin (DMOL), and monomethylluciferin (MMOL) with recombinant wild-type and mutant (His433Tyr) luciferases from the *Luciola mingrelica* firefly were determined by fluorescent titration. The protonated effectors were bound by the wild-type and mutant luciferases better than the nonprotonated ones. The affinity of DMOL for the mutant luciferase was higher than for the wild-type luciferase at alkaline pH, whereas the affinity of MMOL was higher at all pH values studied. The fluorescence emission and excitation spectra of DMOL and MMOL in buffer solution (pH 7.8) were obtained in the absence and presence of luciferase. The fluorescence maxima of DMOL and MMOL complexes with luciferase were 20 and 100 nm, respectively, shifted to shorter wavelengths as compared to the values in buffer solution. This was explained by nonspecific and specific influence of the protein microenvironment on the fluorescence spectra of DMOL and its specific influence on the MMOL fluorescence spectra.

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*Key words*: firefly luciferase, bioluminescence, fluorescence, luciferin, oxyluciferin, monomethyloxyluciferin, dimethyloxyluciferin

Firefly luciferase catalyzes oxidation of luciferin with oxygen of the air in the presence of ATP and $Mg^{2+}$ [1]. The chemical scheme for the reaction and the emitter structure (of the reaction product oxyluciferin in the singlet electron-excited state) are identical for all luciferases isolated from insects. The main difference is in the color of the induced bioluminescence, which depends on the microenvironment of the emitter located in the active site of the enzyme [2]. According to photophysical concepts about the influence of microenvironment on the luminescence spectra, the observed effects can be divided into nonspecific (general) and specific [2, 3]. The nonspecific effects are characterized by orientational polarizability ($\Delta f$) of the microenvironment, that is a function of dielectric constant of the medium ($\varepsilon$) and the flexibility of the groups in the emitter microenvironment. The higher is $\Delta f$, the greater is the shift of the luminescent maximum $\lambda_{em}$ to the longer wavelengths in the spectrum. The excitation maximum $\lambda_{ex}$ of the emitter, as a rule, does not change. Specific effects are observed on formation of hydrogen or acid–base bonds or charge transfer complexes between functional groups of the emitter and microenvironment molecules. This is associated with a change in the excitation maximum of the emitter, i.e., a change in its structure [3].

It is difficult to distinguish these effects in real bioluminescent systems. Thus, the single mutation His433Tyr of the *Luciola mingrelica* luciferase [4] at the pH optimum (pH 7.8) of the enzyme activity resulted in a shift of the bioluminescence $\lambda_{em}$ to longer wavelengths (from 566 nm for the wild-type luciferase to 606 nm for the mutant form). This was explained by an increase in the flexibility of protein residues in the emitter microenvironment (increase in $\Delta f$) on one hand and by specific effects (shift of equilibria between different forms of the emitter) on the other hand.

Luciferin and oxyluciferin have pronounced fluorescence; therefore, fluorescent methods are promising for investigation of the mechanism of the interaction of the substrate ($LH_2$) with luciferase [5]. Oxyluciferin in aque-

*Abbreviations*: $K_s$) dissociation constant of luciferase complex with effectors; $LH_2$) luciferin; OL) oxyluciferin; DMOL) dimethyloxyluciferin; MMOL) monomethyloxyluciferin; $\lambda_{em}$, $\lambda_{ex}$) wavelengths of fluorescence emission and excitation spectra maximums, respectively.

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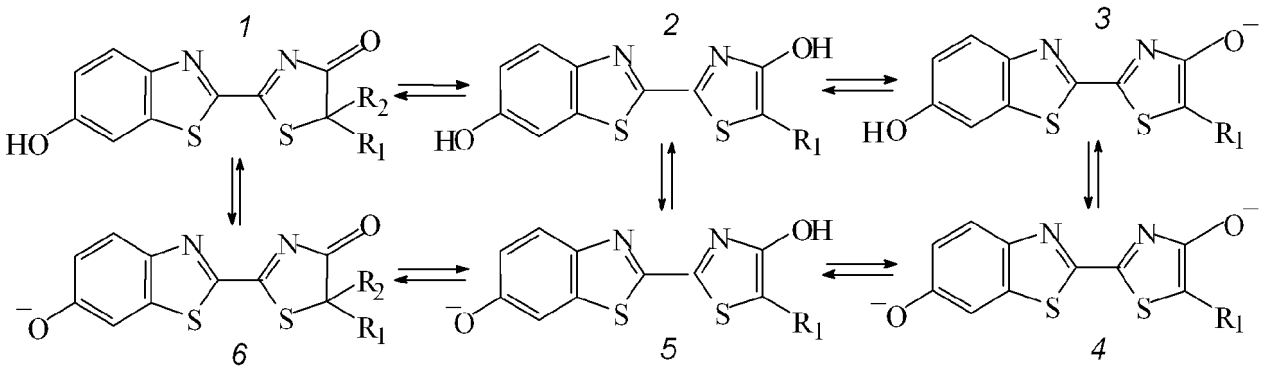

**Fig. 1.** Different forms of oxyluciferin and its analogs: oxyluciferin $R_1 = R_2 = H$; monomethyloxyluciferin $R_1 = CH_3$, $R_2 = H$; dimethyloxyluciferin $R_1 = R_2 = CH_3$.

ous solutions is unstable; therefore, in the present work its more stable analogs were used: dimethyloxyluciferin (DMOL) and monomethyloxyluciferin (MMOL). DMOL has two methyl groups in the thiazole cycle and can exist only as the forms *1* and *6* (Fig. 1). MMOL has one methyl group in the thiazole cycle and can like OL exist in the forms *2-4* (Fig. 1). DMOL is a competitive inhibitor with respect to $LH_2$ and similarly to $LH_2$ forms with luciferase a complex characterized by quenching of the protein fluorescence by the static mechanism [7]. This suggests the binding of oxyluciferin analogs DMOL and MMOL in the active site of luciferase, and, thus, they can be used as fluorescent labels to study the effect of the emitter microenvironment in the active site of the wild-type luciferase and its mutant form with the single substitution His433Tyr on luminescence spectra of the emitter.

## MATERIALS AND METHODS

DMOL and MMOL synthesized by the method of Weiss [8] and luciferin synthesized as described in [9] were used. Luciferase of the *L. mingrelica* firefly was isolated from *Escherichia coli* (strain LE 392) cells carrying the pLR plasmid with the gene of the wild-type or mutant (with His433Tyr substitution) luciferase, purified as described in [10], and stored at –70°C. Measurements were performed in 0.05 M Tris-acetate buffer solution supplemented with 2 mM EDTA, 10 mM $MgSO_4$, and 6 mM dithiothreitol (DTT), which was prepared using distilled deionized water obtained with a Milli-Q apparatus (Millipore, USA). Initial DMOL and MMOL solutions were prepared in freshly distilled dioxane. Fluorescence was measured with an LS 50B spectrofluorimeter (Perkin Elmer, England) using a triangle cuvette of 1.5 ml volume to diminish the internal filter effect. The error in the spectral measurement was ±2.5 nm. The spectra were processed by the Gauss method using the Microcal Origin 6.0 Professional program.

Dissociation constants of complexes of the effectors ($LH_2$, DMOL, and MMOL) with luciferase ($K_s$) were determined at pH 6.5-9.0 by fluorescence titration method measuring the quenching of the protein fluorescence. The protein fluorescence spectra ($\lambda_{ex}$ = 290 nm) were recorded at 300-400 nm on addition to 1 ml of 1 μM luciferase solution of 0.5-μl portions of 10 mM solution of the effector. Using the Stern–Volmer equation [3] the $K_s$ values were determined from the plot of $[(I_0/I) - 1]$ versus the effector concentration $[Q]$, where $I_0$ and $I$ are intensities of luciferase fluorescence in the absence and presence of the effector, respectively [3].

The fluorescence and excitation spectra of DMOL and MMOL were obtained by addition of 2 μl of 10 mM solution of the effector to 1 ml of buffer solution or the enzyme solution containing either 50 μM wild-type or 35 μM mutant luciferase. The measurements were also performed for DMOL using the wild-type luciferase solutions with the concentration from 6.3 to 50 μM. The excitation and emission wavelengths are shown in captions to the figures and table.

The polarization degree of the effector fluorescence was determined by the formula:

$$P = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh}),$$

where $G = I_{hv}/I_{hh}$, and $I$ is the fluorescence intensity with the vertical (v) or horizontal (h) position of polarizers for the exciting (first index) and the emitted (the second index) light [3]. Concentrations of the effectors and luciferase were chosen corresponding to complexing of 50% of the effector with the enzyme: to 1 ml of 50 μM solution of the wild-type luciferase, 2 μl of 10 mM DMOL or MMOL solution was added; to 25 μM of the enzyme solution 1 μl of 10 mM solution of $LH_2$ was added. And $\lambda_{ex}$ was 327, 485, and 392 nm for $LH_2$, DMOL, and MMOL, respectively.

The orientation polarizability of the solvent was calculated by the formula:

$$\Delta f = [(\varepsilon - 1)/(2\varepsilon + 1)] - [(n^2 - 1)/(2n^2 + 1)],$$

where ε is the dielectric constant and $n$ is the refraction index [3]. For calculation, the known values of ε and $n$ were used [11].

## RESULTS AND DISCUSSION

**The pH dependence of dissociation constants of effector complexes with recombinant wild-type and mutant luciferases.** The dissociation constants for the $LH_2$, DMOL, and MMOL complexes with the wild-type and mutant luciferases were obtained by fluorescence titration at pH 6.5-9.0 (Fig. 2). The $K_s$ values for the wild-type luciferase complexes with DMOL and MMOL were higher than for its complex with $LH_2$. The additional methyl groups in the DMOL and MMOL molecules seemed to be responsible for some steric hindrances for their binding to the enzyme. The affinity of DMOL for the mutant luciferase was increased at alkaline pH values, whereas the affinity of MMOL was increased at all pH values studied. The mutant luciferase was earlier shown to have more flexible structure in the active site in comparison with the wild-type enzyme [4]. Therefore, the binding of DMOL and MMOL with the mutant luciferase met less pronounced steric hindrances than their binding with the wild-type luciferase. In the studied pH interval, these compounds were present either protonated or deprotonated in the phenol group (for $LH_2$ p$K$ = 8.5 [12] and for DMOL and MMOL p$K$ = 7.8 [6, 7]). The protonated effectors bound better with both the wild-type and mutant luciferase (Fig. 2).

**Fluorescence spectra of DMOL.** Spectra were recorded in buffer solution (pH 7.8) in the absence and presence of the wild-type and mutant luciferases (Fig. 3). On increasing the concentration of the wild-type luciferase from 0 to 50 μM, the fluorescence maximum of DMOL shifted to shorter wavelengths, and the fluorescence intensity increased. Thus, $\lambda_{em}$ for DMOL moved from 639 nm in the buffer solution to 619 nm in the presence of luciferase when 50% of DMOL was complexed with the enzyme. The shift was still more pronounced in the presence of the mutant luciferase (to 614 nm), and the fluorescence intensity was also higher (Fig. 3). In both cases, $\lambda_{ex}$ in the DMOL excitation spectra on the binding with luciferase did not change. This suggested that, similarly to the buffer solution, the phenolate form of DMOL was the emitting species (Fig. 1, form *6*) [3, 4].

The shift of $\lambda_{em}$ to shorter wavelengths without changes in $\lambda_{ex}$, as noted above is explained by a nonspecific effect of the chromophore microenvironment namely by decrease in the orientational polarizability. We measured $\lambda_{em}$ for DMOL in solvents with different orientational polarizability (table) and found that for DMOL a decrease in the solvent $\Delta f$ really resulted in $\lambda_{em}$ shift to shorter wavelengths. Consequently, the microenvironment of the DMOL in complex with luciferase was characterized by lower orientational polarizability than in aqueous solution. Low orientational polarizability is a specific feature of highly hydrophobic solvents [3], and an increase in the microenvironment hydrophobicity usually results in the shift of the emitter $\lambda_{em}$ to shorter wave-

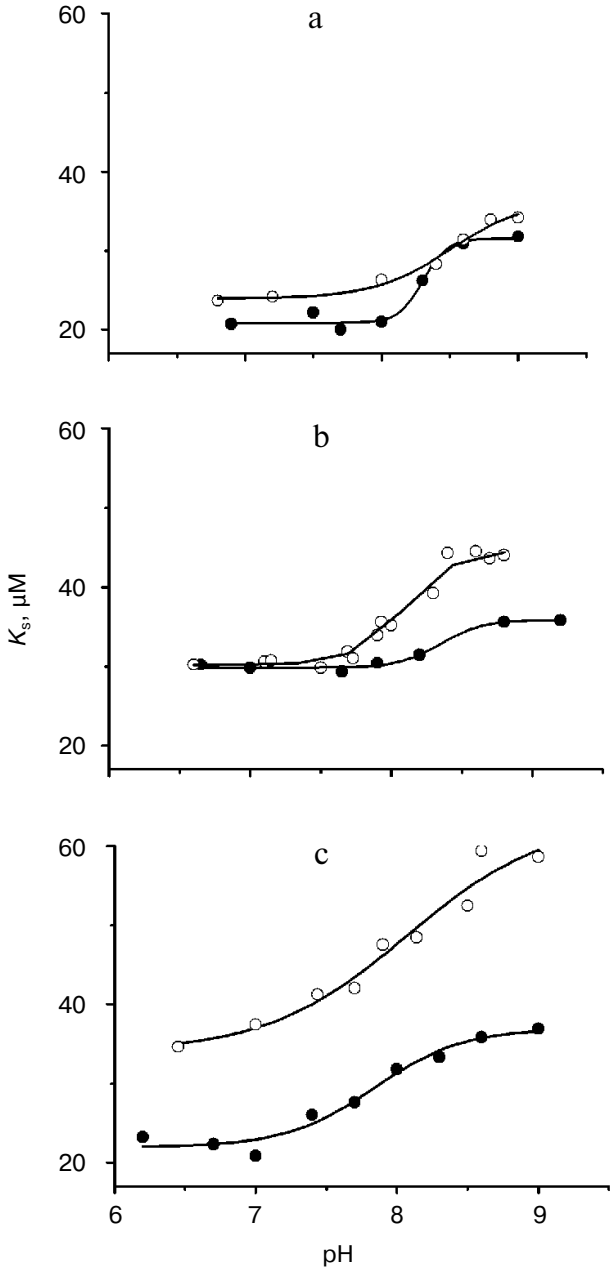


**Fig. 2.** The pH dependence of dissociation constants of wild-type (light dots) and mutant (dark dots) *L. mingrelica* luciferase complexes with luciferin (a), dimethyloxyluciferin (b), and monomethyloxyluciferin (c). Conditions: 0.05 mM Tris-acetate buffer, 2 mM EDTA, 10 mM $MgSO_4$, 6 mM DTT, 20°C, $\lambda_{ex}$ = 290 nm.

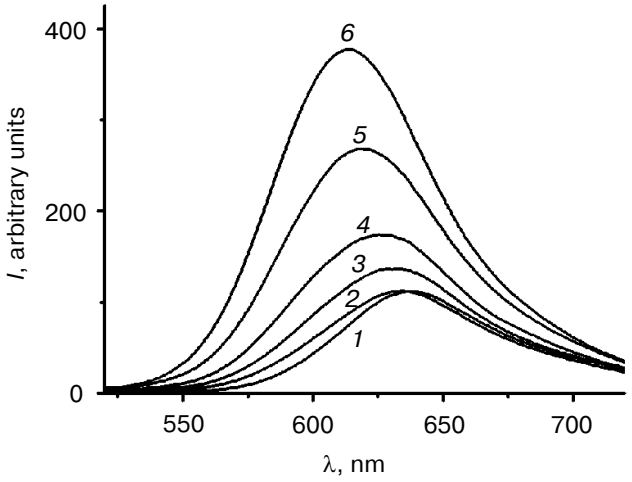


**Fig. 3.** Fluorescence spectra of dimethyloxyluciferin in buffer solution (*1*) and in the presence of wild-type (*2-5*) and mutant (*6*) luciferases at pH 7.8 and $\lambda_{ex}$ = 485 nm. Concentrations (μM): DMOL, 20; wild-type luciferase, 6.3, 12.5, 25, and 50 (*2-5*, respectively); mutant luciferase, 35 (*6*). Other conditions are the same as in Fig. 2.

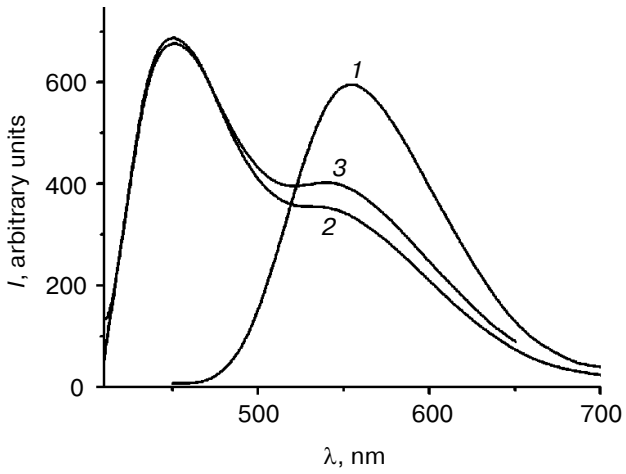


**Fig. 4.** Fluorescence spectra of monomethyloxyluciferin in buffer solution (*1*) and in the presence of wild-type (*2*) and mutant (*3*) luciferases at pH 7.8 and $\lambda_{ex}$ = 392 nm. Concentrations (μM): MMOL, 10; wild-type luciferase, 50; mutant luciferase, 35. Other conditions are the same as in Fig. 2.

lengths. The experimental findings suggested that the microenvironment of the DMOL in complex with luciferase was more hydrophobic than in the buffer solution. This was also confirmed by data on a rather high hydrophobicity of the luciferase active site [13, 14]. The more pronounced blue shift of $\lambda_{em}$ for DMOL on the binding to the mutant luciferase seemed to indicate an increase in the hydrophobicity of the DMOL microenvironment in the mutant protein as compared to the wild-type luciferase.

**Fluorescence spectra of MMOL.** The spectra were recorded in buffer solution at pH 7.8 in the absence and presence of the wild-type and mutant luciferases (Fig. 4). In the buffer solution, MMOL had a symmetric fluorescence spectrum with $\lambda_{em}$ = 550 nm typical for the MMOL dianion (Fig. 1, form *4*), as shown earlier [6]. In the presence of luciferase, a complex spectrum was recorded with $\lambda_{em}$ = 450 nm and a shoulder on the long wavelength region. Gaussian multi-peak fitting of this spectrum allowed us to identify two components: $\lambda_{em}$ = 550 nm corresponding to the free MMOL molecule not bound with the protein, and $\lambda_{em}$ = 450 nm corresponding to MMOL in the complex with luciferase. Consequently, the binding with luciferase was associated with a 100 nm shift of the MMOL $\lambda_{em}$ to shorter wavelengths. Such a large shift in the $\lambda_{em}$ cannot be explained only by influence of the hydrophobic microenvironment of MMOL in the complex with luciferase. It seems that in the MMOL–luciferase complex the structure of the emitter is changed.

To test this hypothesis, we measured excitation spectra at pH 7.8 for MMOL in buffer solution and in the presence of luciferase (Fig. 5). For MMOL in the buffer solution (Fig. 5a) at $\lambda_{em}$ = 550 nm the excitation spectrum with $\lambda_{ex}$ = 430 nm was observed, which was a superposition of spectra with $\lambda_{ex}$ equal to 400 and 440 nm corresponding to MMOL mono- and dianion (Fig. 1, forms *3* and *4*). For MMOL in the luciferase solution (Fig. 5b) at $\lambda_{em}$ = 450 nm the excitation spectrum with $\lambda_{ex}$ = 392 nm was a superposition of spectra with $\lambda_{ex}$ equal to 375 and 400 nm which corresponded to the protonated

Fluorescence maxima of dimethyloxyluciferin in solvents with different orientational polarizability

| Solvent | ε [11] | *n* [11] | Δ*f* | $\lambda_{em}$, nm |
|---|---|---|---|---|
| Water | 78.3 | 1.33 | 0.32 | 639 |
| Ethanol | 24.55 | 1.36 | 0.29 | 623 |
| Acetone | 20.56 | 1.36 | 0.28 | 622 |
| Methylethylketone | 18.51 | 1.38 | 0.27 | 622 |
| Dichloromethane | 8.93 | 1.42 | 0.22 | 604 |

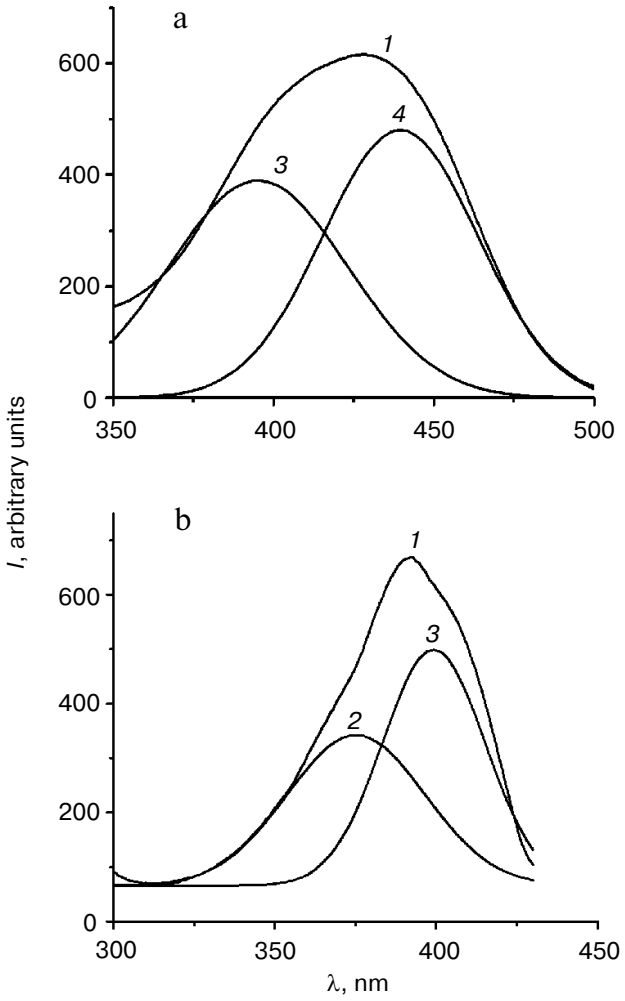


**Fig. 5.** Excitation spectra of monomethyloxyluciferin in buffer solution, $\lambda_{em}$ = 550 nm (a), and in solution of the wild-type luciferase, $\lambda_{em}$ = 450 nm (b). The experimental spectrum (*1*) and spectra obtained by Gaussian multi-peak fitting corresponding to the protonated form (*2*), monoanion (*3*), and dianion (*4*). Conditions are the same as in Fig. 4.

form and monoanion of MMOL (Fig. 1, forms *2* and *3*). Thus, on the binding with luciferase, the equilibrium between the different MMOL forms shifted from di- and monoanions to monoanion and protonated form. This could be caused by specific interactions of polar groups of the MMOL molecule with polar amino acid residues of the luciferase active site [14-16] resulting in the increase of the apparent values of p*K* of the phenol and hydrothiazole groups of MMOL.

Note that the excitation spectra of MMOL in solution of luciferase obtained at pH 7.8 were similar to the excitation spectra of MMOL in buffer solution at pH 6.8. Thus, the MMOL molecule may be considered as a sensitive indicator to characterize an apparent micro-pH in the active site of luciferase near the hydroxythiazole group.

**Polarization of the fluorescence of the emitter.** Polarized fluorescence is an essential characteristic of the interaction of the effector with the protein. It was shown [12] that the light-induced excitation of luciferin in the complex with luciferase resulted in dissociation of this complex that prevented polarization of the luciferin fluorescence. The polarization of DMOL and MMOL fluorescence in the complexes with luciferase was 0.26 and 0.32, respectively. Consequently, the DMOL and MMOL complexing with the protein impedes free rotation of the electron-excited molecules of the emitter.

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