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Quenching of the Fluorescence of Tyr and Trp Residues of Firefly Luciferase from *Luciola mingrelica* by the Substrates

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Abstract—Luciferase of the firefly *Luciola mingrelica* is characterized by fluorescence of not only the unique Trp residue (λ_{em} = 340 nm), but also that of Tyr residues (λ_{em} = 308 nm). Quenching of the intrinsic fluorescence of the luciferase by its substrates luciferin and ATP (AMP) has been studied. Luciferin (LH_2) quenches Trp fluorescence more efficiently than the fluorescence of Tyr residues. Two centers of quenching of Tyr fluorescence by ATP have been found corresponding apparently to the allosteric and active sites of the luciferase with $K_{s(ATP)}$ = 20 and 110 μM, respectively. The influence of one substrate on the affinity of luciferase to the second was investigated using fluorescence. ATP (AMP) binding to the allosteric sites of the luciferase significantly affects the affinity of luciferase to LH_2. Formation of the complex between the luciferase and LH_2 affects the affinity of both allosteric and active sites of the luciferase to ATP (AMP). The observed effects are probably connected with conformational changes in the luciferase molecule upon its interaction with the substrates.

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Fluorescence methods are widely used for investigation of the interaction of enzymes with their substrates and other effectors that are capable of quenching the intrinsic fluorescence of proteins, which is mainly from the fluorescence of tryptophan and tyrosine residues [1, 2]. Firefly luciferase catalyzes oxidation of luciferin (LH_2) by oxygen in the presence of ATP and Mg^{2+} yielding the electronically excited product oxyluciferin, whose transition into the ground state is accompanied by the emission of visible light [3]. Luciferase of the firefly *Luciola mingrelica* contains one Trp residue and 19 Tyr residues [4].

To date investigation of the interaction of the luciferase with effectors has used the fluorescence of the Trp residue as the fluorescent probe (λ_{em} = 340 nm), since the intensity of the Trp fluorescence decreases in the presence of the substrates (LH_2, ATP) [5-10]. In the presence of LH_2, the fluorescence quenches according to the static type, since the lifetime of the Trp residue in the excited state does not change [8]. In the luciferase–LH_2 complex, effective energy transfer from Trp to LH_2 was observed [7, 9, 10]. The second substrate (ATP) affects the Trp fluorescence, being employed only in concentrations significantly exceeding the K_m value for ATP (130 ± 30 μM) [5]. In the presence of a large excess of ATP (7 mM), the lifetime of the Trp residue in the excited state is reduced, this indicating the dynamic type of quenching [7, 9].

The fluorescence of the Tyr residues of firefly luciferase has not been investigated until now. According to the literature, significant tyrosine fluorescence (λ_{em} = 304-308 nm) is observed in proteins where the number of Tyr residues exceeds the number of Trp residues [11-17]. Since the luciferase of the firefly *L. mingrelica* contains 19 Tyr residues and only one Trp residue, the contribution of the tyrosine component into the intrinsic fluorescence of the luciferase can be significant. It is known that even slight changes in the three-dimensional protein structure can result in quenching of the Tyr fluorescence due to the appearance of proton acceptors in the proximity to Tyr, which promote ionization of its hydroxyl group in the original or excited states, energy transfer from Tyr to Trp

Abbreviations: K_s) dissociation constant of the luciferase complex with an effector; R_0) Forster radius (distance between donor and acceptor when efficiency of energy transfer is 50%); K_m) Michaelis constant; LH_2) luciferin; λ_{em}, λ_{ex}) positions of the maxima of the fluorescence and excitation spectra, respectively.

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(R_0 = 14 Å), and formation of hydrogen bonds with the participation of the hydroxyl groups of Tyr residues [1, 12, 15]. Thus, the use of Tyr residues as fluorescence probes can be a promising approach for the investigation of the changes in the structure of firefly luciferase upon its interaction with the substrates and other effectors.

Firefly luciferase consists of two domains: the large N-domain and the small C-domain are connected by a flexible loop of disordered structure [18]. The formation of the complex with the substrates results in the rotation of the C-domain and its approach to the N-domain, this changing the conformation of the connecting loop and resulting finally in the formation of the active site [4]. However, it is not known which of the substrates (LH_2 or ATP) causes the observed alteration.

The goal of the present study was to investigate fluorescence properties of the Tyr residues of the luciferase from the firefly *L. mingrelica* in the absence and in the presence of the substrates and to study the effect of one substrate on the affinity of the luciferase to the second one using the fluorescence method. This allowed us to obtain information concerning the role of each substrate in the conformational changes occurring during the formation of the ternary enzyme–substrate complex luciferase–LH_2–ATP. To investigate the fluorescence properties of the luciferase in the presence of both substrates (LH_2 and ATP), we used AMP as the model of ATP, since the adenosine fragment of the molecule is the most important component for the formation of the enzyme–substrate complex [7, 19].

MATERIALS AND METHODS

In the present study, we used luciferin synthesized as described in work [20], adenosine-5′-triphosphate (ICN, USA), and adenosine-5′-monophosphate (Sigma, USA). Luciferase of the firefly *L. mingrelica* was isolated from the cells of *Escherichia coli* (strain LE 392) bearing the pLR plasmid with the gene of luciferase according to the method described previously [21] and stored at −70°C. Experiments were performed at 20°C in 0.05 M Tris-acetate buffer containing 2 mM EDTA, 10 mM $MgSO_4$, 1 mM dithiothreitol (DTT), pH 7.8 (optimal pH for the luciferase activity), prepared using Milli-Q deionized water (Millipore, USA). Stock solutions of LH_2, ATP, and AMP were prepared using the indicated buffer solution. Fluorescence was measured using an LS 50B spectrofluorimeter (Perkin Elmer, England) with a 1.5-ml triangular cuvette for reducing internal filter effects. The fluorescence spectra of the luciferase were recorded using λ_{ex} = 275 and 295 nm, and the excitation spectra were recorded at λ_{em} = 308 and 340 nm. The measurement error in wavelength constituted ±2.5 nm. The spectra were analyzed by Gauss functions using the program Microcal Origin 6.0 Professional.

The fluorescence of the luciferase was titrated by the addition of an effector (by 0.5-1.0 μl of 10 mM solution) to 1 ml of 1 μM luciferase. While studying the Tyr fluorescence, the intrinsic fluorescence emission spectra of the luciferase were recorded in the range 290-380 nm using λ_{ex} = 275 nm, and the fluorescence intensity of the component with λ_{em} = 308 nm was determined after the decomposition of the experimental spectrum into two components according to the Gaussian multi-peak-fitting into the spectra. While studying the Trp fluorescence, the intrinsic fluorescence spectra of the luciferase were recorded in the range 305-400 nm using λ_{ex} = 295 nm, and the fluorescence intensity was determined at λ_{em} = 340 nm. The data on quenching of the Tyr and Trp fluorescence were analyzed by nonlinear regression analysis (Microcal Origin 6.0 Professional) using the Stern–Volmer equations [1] for complete (1) and partial (2) fluorescence quenching:

$$I = I_0/(1 + [Q]/K_s), \quad (1)$$

$$I = I_0(1 - f[Q]/(Q + K_s)), \quad (2)$$

where [Q] is the concentration of the quencher; I_0 and I are the fluorescence intensities in the absence and in the presence of the quencher, respectively; K_s is the dissociation constant of the luciferase complex with the effector; and f is the fraction of the original fluorescence accessible to quenching.

The parameters of quenching of the luciferase fluorescence by the second effector in the presence of the first effector were determined by the sequential fluorescent titration of the luciferase. In the case of the use LH_2 as the first effector, the luciferase was first titrated with LH_2 until the original fluorescence reduced two-fold, which was observed in the presence of 25 μM LH_2. Under these conditions, 70% of the luciferase formed a complex with LH_2. The resulting solution was then titrated with the second effector (AMP). When AMP was used as the first effector, the luciferase was titrated with AMP until its concentration reached the $K_{m(ATP)}$ (130 μM). Under these conditions, approximately 50% of the luciferase formed the complex with AMP. Then the complex was titrated with luciferin.

The microenvironment of the Tyr residues in the tertiary structures of the luciferase [4] in the absence and in the presence of both substrates (LH_2 and ATP) was analyzed using the program Swiss-PdbViewer, v. 3.7 (SP5).

RESULTS AND DISCUSSION

Intrinsic fluorescence spectra of the luciferase. The spectra of luciferase intrinsic fluorescence were obtained using λ_{ex} 275 and 295 nm (Fig. 1). Gaussian analysis of the spectra showed that they result from the superposition

of two components with λ_{em} of 308 ± 3 nm (Fig. 1, curve *2*) and 340 ± 2 nm (Fig. 1, curve *3*) with the fluorescence intensity of the components depending on the λ_{ex} value. As demonstrated earlier [5-8], fluorescence with λ_{em} 340 nm arises from the only Trp residue (Trp419) of the luciferase. Fluorescence with λ_{em} 308 nm is characteristic for Tyr residues of some proteins [11, 12, 16]. In the case of λ_{ex} = 275 nm, the difference in the fluorescence intensity of the components was minimal (Fig. 1a), since under these conditions, both Tyr and Trp residues absorb [1]. When λ_{ex} = 295 nm, the Trp residue mainly absorbs, and consequently, fluorescence with λ_{em} = 340 nm (Fig. 1b) prevails. This is also confirmed by the difference in the excitation spectra for the fluorescence with λ_{em} of 308 and 340 nm.

Thus, it was first demonstrated that the firefly luciferase is characterized by fluorescence of not only the Trp residue (λ_{em} = 340 nm), but also Tyr residues (λ_{em} = 308 nm). Comparing the fluorescence spectrum of the luciferase with the spectrum of a model aqueous solution containing tryptophan and tyrosine (1 : 18) [1], it was observed that under λ_{ex} = 275 nm the contribution of the Tyr fluorescence in the luciferase molecule is significantly lower than in the model mixture. Apparently, fluorescence of most Tyr residues in the luciferase is quenched, as in most proteins [1].

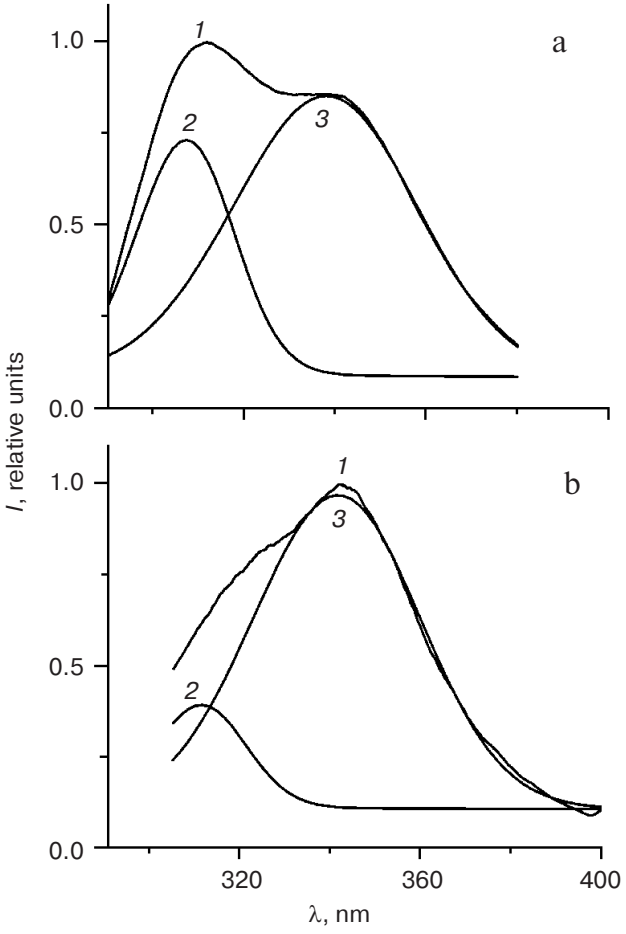

Fig. 1. Fluorescence spectra of the luciferase under λ_{ex} = 275 nm (a) and λ_{ex} = 295 nm (b). The experimental spectrum (*1*) was decomposed by Gaussian multi-peak-fitting into the spectra corresponding to Tyr (*2*) and Trp (*3*) fluorescence. The spectra were recorded at 20°C in 0.05 M Tris-acetate buffer containing 2 mM EDTA, 10 mM $MgSO_4$, 1 mM DTT, pH 7.8.

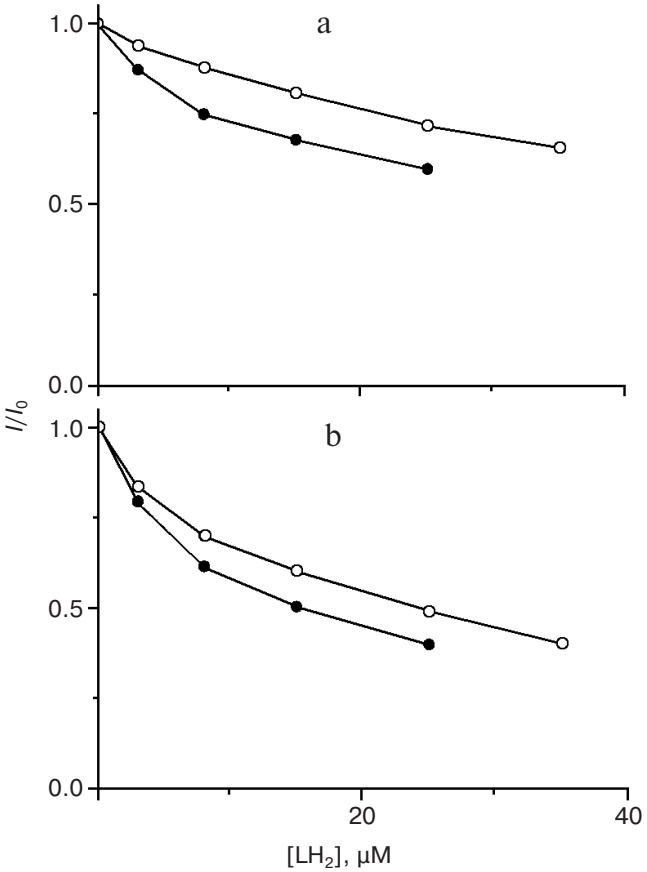

Fig. 2. Dependence of the fluorescence intensity of Tyr (a) and Trp (b) residues of the luciferase on LH_2 concentration in the absence of AMP (filled circles) and in the presence of 130 μM AMP (open circles). The conditions are given in the legend to Fig. 1.

Analysis of the microenvironment (5 Å) of Tyr residues in the three-dimensional structure of the luciferase [4] showed that the hydroxyl groups of the residues Tyr 31, 35, 54, 57, 229, 262, 403, and 428 are involved in the formation of the hydrogen bonds, and consequently, these residues do not fluoresce. The residue Tyr11 is located 5.8 Å from Trp419. Since for the Tyr–Trp pair the R_0 value is 14 Å [1], there is a high probability that the fluorescence of Tyr11 is quenched due to energy transfer to Trp419. The residues Tyr 17, 110, 257, 282, 427, 446, 449, and 503 are surrounded by proton acceptors that can contribute to partial or complete fluorescence quenching due to the ionization of the hydroxyl groups of the indicated residues. In the absence of substrates, Tyr98 and Tyr342 are not involved in the listed interactions and they are likely to make the main contri-

Table 1. K_s and f values for the complexes of luciferase with luciferin calculated by fluorescence titration of Tyr and Trp residues of the luciferase in the absence and in the presence of 130 μM AMP

Fluorescing residue	E		E · AMP	
	K_s, μM	f, %	K_s, μM	f, %
Tyr	10 ± 1	55 ± 2	30 ± 1	61 ± 1
Trp	7 ± 1	75 ± 4	20 ± 1	83 ± 2

bution to the fluorescence spectrum with λ_{em} = 308 ± 3 nm.

Quenching of luciferase fluorescence in the presence of LH_2. The dependence of the fluorescence intensity of Tyr and Trp residues of the luciferase on LH_2 concentration was investigated (Fig. 2). In the presence of LH_2, quenching of the Tyr and Trp fluorescence was observed. The experimental data were processed by non-linear regression analysis according to Eqs. (1) and (2). Equation (1) does not fit the experimental data exactly. The data on the fluorescence quenching of Tyr and Trp residues were adequately described by Eq. (2) for partial quenching with correlation coefficient exceeding 0.99. Using Eq. (2), the values of the parameters f (the fraction of the fluorescence accessible for quenching) and K_s for the complex of the luciferase with LH_2 were determined (Table 1). The f values constitute 55 and 75% for the Tyr and Trp residues, respectively. Consequently, LH_2 quenches the fluorescence of the Trp residue more efficiently than that of the Tyr residues. The K_s values for the complex of the luciferase with LH_2 determined from the data on quenching of the Trp and Tyr fluorescence constitute 7 ± 1 and 10 ± 1 μM, respectively (Table 1), which is in agreement with the K_m value (11.5 ± 2.7 μM) obtained previously from kinetic experiments [5].

Quenching of luciferase fluorescence in the presence of ATP and AMP. The dependence of the fluorescence intensity of Tyr and Trp residues of the luciferase on concentration of ATP and AMP (0-300 μM) was investigated (Fig. 3). It was first demonstrated that ATP (AMP) are efficient quenchers of the Tyr fluorescence. The non-linear regression analysis of the experimental data according to Eqs. (1) and (2) demonstrated that under high concentrations of the quencher (35-300 μM), the experimental data were described reasonably well by Eq. (1) for the complete quenching or by Eq. (2) assuming that f = 100%. Under low concentrations of the quencher (less than 35 μM), the data are described by Eq. (2) for partial fluorescence quenching (f ~ 40%). Within the concentration range of ATP and AMP 35-300 μM, the K_s values are 110 ± 7 and 150 ± 8 μM, respectively (Table 2). This is in agreement with the $K_{m(ATP)}$ value (130 ± 30 μM) obtained previously from kinetic experiments [5]. Thus, binding of ATP (AMP) in the active site of the luciferase results in the complete quenching of fluorescence of Tyr residues.

At low concentrations of ATP and AMP (less than 35 μM), the K_s values constituted 20 ± 3 and 20 ± 1 μM, respectively (Table 2), this being significantly lower that the K_m value for ATP. Under these conditions, residual Tyr fluorescence was observed (f ~ 40%). Previously it was shown that besides the substrate-binding site, the firefly luciferase has allosteric sites for binding ATP and its

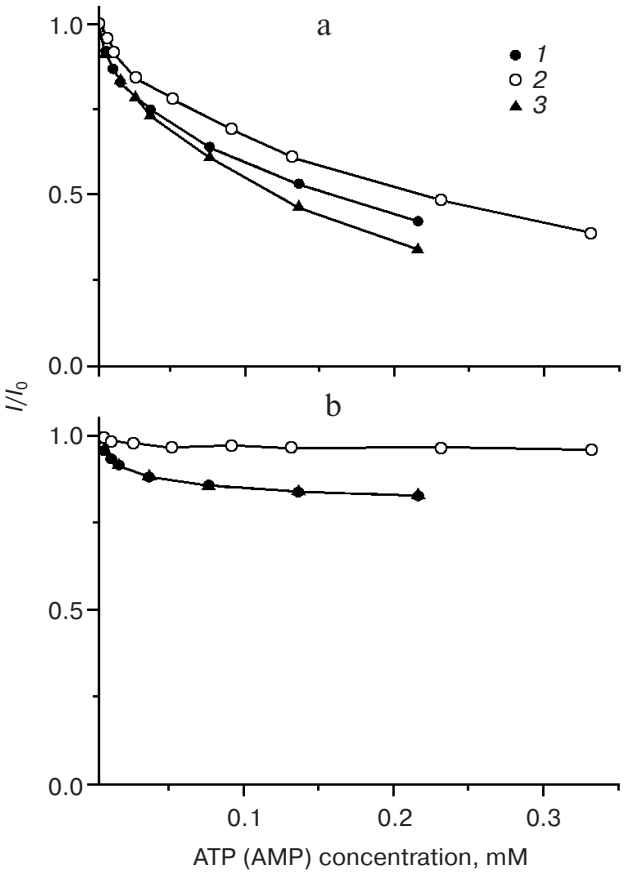

Fig. 3. Dependence of the fluorescence intensity of Tyr (a) and Trp (b) residues of luciferase on AMP concentration in the absence of LH_2 (*1*) and in the presence of 25 μM LH_2 (*2*) and on ATP concentration (*3*). The conditions are described in the legend to Fig. 1.

Table 2. K_s and f values for the complexes of luciferase with ATP and AMP calculated by fluorescence titration of Tyr and Trp residues of the luciferase in the absence and in the presence of 25 μM luciferin

Quencher	Quencher concentration, μM	E		E · LH_2	
		K_s, μM	f, %	K_s, μM	f, %
Tyr					
AMP	0-35	20 ± 1	39 ± 1	34 ± 1	37 ± 1
	35-300	150 ± 8	100 ± 5	210 ± 5	100 ± 1
ATP	0-35	20 ± 3	40 ± 2	—	
	35-300	110 ± 7	100 ± 5		
Trp					
AMP	0-300	20 ± 1	18 ± 1	no quenching	
ATP	0-300	20 ± 2	18 ± 2	—	

analogs [22, 23]. The K_m values for these two ATP binding sites were 20 and 110 μM [23], this being in agreement with our results. Thus, fluorescence of Tyr residues in the luciferase is a sensitive marker of structural changes in the enzyme on its interaction with ATP (AMP). It should be noted that for the active site of the luciferase the $K_{s(ATP)}$ value is a little lower (110 μM) than $K_{s(AMP)}$ value (150 μM), while in the case of the allosteric sites, the K_s values for ATP and AMP are equal. This is presumably connected with the fact that the active site of the luciferase is highly specific to both the adenine part and the phosphate group number of the ATP molecule, while the allosteric binding sites do not possess this specificity.

Thus, the results demonstrate that in the presence of the substrates, a significant quenching of the Tyr fluorescence in the luciferase is observed. Analysis of tertiary structures of the luciferase in the absence and in the presence of both substrates (LH_2, ATP) showed that the microenvironment of Tyr residues 98 and 342 changes in the presence of the substrates, their hydroxyl groups being involved in the formation of the hydrogen bonds. In the complex with both substrates, Tyr342 is directly involved in the formation of the hydrogen bond with ATP (Fig. 4). Consequently, redistribution of the hydrogen bonds can be one of the reasons for quenching of the Tyr fluorescence of the luciferase on the formation of the enzyme–substrate complex.

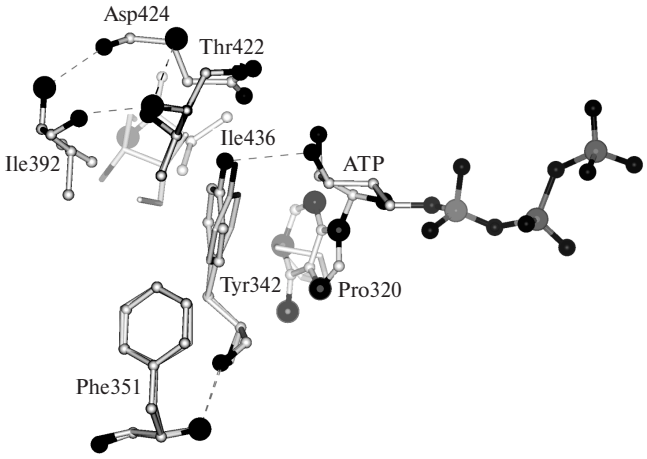

Fig. 4. Microenvironment of Tyr342 in the luciferase of the firefly *L. mingrelica*: free enzyme (rod-like model) and in the complex with the substrates (sphere-rod model).

The data obtained by the fluorescence titration of the Trp residue within the concentration range of ATP (AMP) of 0-300 μM were described better by Eq. (2). The f value constituting 18% indicates a low efficiency of Trp fluorescence quenching. The K_s values of 20 ± 2 μM (Table 2) calculated from Eq. (2) for the complexes of the luciferase with ATP (AMP) are significantly lower than the $K_{m(ATP)}$ value and agree with the K_s values calculated from the quenching of the Tyr fluorescence in the presence of low concentrations of ATP (AMP). Consequently, the slight quenching of Trp fluorescence can be due to the binding of ATP (AMP) to the allosteric sites of the luciferase. The binding of ATP (AMP) in the active site of the luciferase does not affect the fluorescence of the Trp residue, which supports previously obtained results [5, 7, 9].

Influence of AMP on affinity of luciferase to LH_2. The experimental data presented above demonstrate that the binding of one of the substrates to the luciferase significantly affects the fluorescence of the Tyr and Trp residues of the luciferase. The binding of LH_2 mostly influences the Trp fluorescence, while the binding of ATP affects the Tyr fluorescence. The observed fluorescence quenching can be due to the conformational changes in the luciferase molecule caused by the binding of the substrates. In this connection, it would be interesting to estimate the influence of the binding of one substrate on the K_s value of the second substrate and on the efficiency of the fluorescence quenching of the enzyme by the second substrate.

To solve this problem, the dependences of the fluorescence intensity of Tyr and Trp residues of the luciferase on LH_2 concentration were studied in the presence of 130 μM ATP (Fig. 2). Under these conditions, 90% of the allosteric sites and 50% of the active sites of the luciferase are occupied by ATP (AMP). As demonstrated above, the binding of ATP (AMP) in the active site results in the complete quenching of the Tyr fluorescence. Therefore, the parameters of quenching of the Tyr fluorescence by luciferin presented in Table 1 are related to the complex of the luciferase with AMP (E–AMP) bearing AMP in the allosteric site, while the parameters of the Trp fluorescence quenching concern all enzyme species in the indicated mixture. The *f* values of the Tyr and Trp residues for the E–AMP complex on quenching by luciferin increased by ~10% compared to these parameters for the free enzyme. According to the results on quenching of the Trp and Tyr fluorescence, the K_s value in the presence of AMP increases about three-fold compared to the free enzyme (Table 1). Consequently, the affinity of the luciferase to LH_2 is mainly affected by the binding of ATP (AMP) to the allosteric sites of the luciferase.

Influence of LH_2 on affinity of luciferase to AMP. The partial fluorescence quenching of the Tyr and Trp residues in the E–LH_2 complex allowed estimation of the K_s and *f* values for the second substrate, AMP (Table 2). The dependences of the fluorescence intensity of the Tyr and Trp residues of the luciferase on AMP concentration were studied in the presence of 25 μM LH_2 (Fig. 3). In the complex E–LH_2, the *f* values for the Tyr residues in the case of quenching by AMP agree with the data obtained for the free enzyme, while the Trp fluorescence becomes inaccessible for quenching by AMP. The K_s values increased 1.7- and 1.4-fold compared to those for the free enzyme within the concentration ranges 0-35 and 35-300 μM, respectively. Consequently, the formation of the complex of the luciferase with LH_2 changes the affinity of both the allosteric and active sites of the luciferase to ATP (AMP).

In conclusion, it should be noted that each of the substrates of the luciferase contributes significantly to the quenching of the intrinsic fluorescence of the luciferase. The K_s values indicate that binding of one substrate hampers subsequent binding of the second substrate. The intrinsic fluorescence probes (Tyr and Trp residues) respond differently to the binding of the substrates to the enzyme. Fluorescence of the Trp residue is a sensitive probe for conformational changes on binding of LH_2 in the active site of the luciferase, and fluorescence of the Tyr residue is a sensor for conformational changes while binding ATP in the both active and allosteric sites of the luciferase.

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