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ORIGINAL RESEARCH

Interaction of aromatic compounds with *Photobacterium leiognathi* luciferase: fluorescence anisotropy study

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ABSTRACT: The time-resolved and steady-state fluorescence techniques were employed to elucidate possible interactions of four aromatic compounds (anthracene, POPOP, MSB and 1,4-naphthalendiol) with bacterial luciferase. Fluorescence spectra and fluorescence anisotropy decays of these compounds were studied in ethanol, water–ethanol solutions and in the presence of bacterial luciferase. Shifts of fluorescent spectra and differences in rotational correlation times are interpreted in terms of weak (hydrophobic) interactions of the molecules with the enzyme. These interactions suggest the feasibility of intermolecular energy transfer by an exchange resonance mechanism with a collision-interaction radius as a way of excitation of these compounds in the reaction catalysed by bacterial luciferase. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: bioluminescence; luciferase; fluorescent compounds; anisotropy decay

INTRODUCTION

Luciferases catalyse reactions in luminous organisms, resulting in light emission. These enzymes take part in the efficient transformation of the energy of chemical reactions into electronic excitation of product molecules (1–3). Luciferase and coupled enzymatic reactions, as well as intact luminous bacteria, are used in ecological monitoring as bioassay systems (4). Hence, the effect of different groups of compounds on luciferase activity is of great interest.

Bacterial luciferase catalyses the oxidation of reduced FMN and long-chain aliphatic aldehyde by molecular oxygen (1–3). The biochemistry of this process is supposed to be known; the reaction proceeds via a number of chemical intermediates. The last of them (luciferase-bound 4α-hydroxyflavin) is responsible for the emission of blue-green light ($\lambda_{max} \sim 490$ nm) (1–3, 5).

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The primary physicochemical processes resulting in formation of the electronically excited emitter in bacterial luciferase reaction are still under discussion. A mechanism has been proposed (6, 7) involving formation of the excited dioxirane molecule as a precursor of the emitter. The excitation energy is considered to migrate from dioxirane onto 4α-hydroxyflavin or some other secondary chromophore available. Another hypothesis suggests involvement of the upper electron-excited state of 4α-hydroxyflavin in the bioluminescent process as a precursor of the bioluminescent emitter (8).

The total energy of the bioluminescent reaction allows a populatation of electron-excited states of higher energy than that of bioluminescent emitter (~22 000/cm): oxidation of reduced FMN and aldehyde liberates 130 kcal/mol energy, corresponding to 45 000/cm (9). Spectral studies of *in vitro* bioluminescence may also predict the existence of the high-energy precursor of bacterial bioluminescence emitter. Lee and his colleagues were the first to observe a blue shift (490 to 475 nm) of *in vitro* bioluminescence maxima caused by addition of lumazine protein to the reaction mixture (2, 10, 11). Later the sensitized luminescence of chemically inert aromatic compounds in a blue region of bioluminescence spectrum of the coupled bioluminescent enzyme system was registered (12–14). The absorption spectra of these compounds did not overlap the bioluminescence spectrum, but the maxima of their fluorescence were found in the range 418–420 nm, i.e. at the shorter wavelength than the bioluminescent one. These results were attributed to the radiationless intermolecular energy transfer from the

upper states of the bioluminescence emitter onto the electron-excited states of the foreign molecules. Two mechanisms could be responsible for this transfer: (a) an inductive resonance (dipole) mechanism, which considers a donor and an acceptor to interact like two dipoles at a distance up to 5 nm; (b) an exchange resonance mechanism with a collision-interaction radius, which assumes closer interactions of a donor and an acceptor at a distance about 1–1.5 nm (13–15). The short-distance interactions may suggest the definite types of intermolecular interactions of aromatic compounds with bioluminescent enzymes (e.g. hydrophobic interactions, coordination binding, etc.). In these cases the fluorescence anisotropy of the aromatic molecule must be sufficiently changed by 'binding' of the massive luciferase molecule (16). This effect was used in current investigations.

The aim of this work was to reveal the mechanism of the interaction of bacterial luciferase with four aromatic compounds: anthracene; 1,4-bis(5-phenyloxasol-2-il)-benzene (POPOP); *p*-bis(*o*-methylsteril)benzene (MSB); and 1,4-naphthalenediol. The absorption spectra of these compounds do not overlap the bioluminescence spectrum. Anthracene, POPOP and MSB were found to exhibit blue-shifted sensitized luminescence in the coupled enzyme bioluminescent system (12–14).

The fluorescence anisotropy decay of these compounds in ethanol and water–ethanol solutions was examined in the presence and absence of bacterial luciferase. Rotational correlation times were found and compared to analyse binding of the compounds with luciferase.

MATERIALS AND METHODS

Reagents

Anthracene (Fluka Chemica, USA), 1,4-bis(5-phenyloxasol-2-il)benzene (POPOP; Eastman, USA), *p*-bis(*o*-methylsteryl)benzene (MSB) and 1,4-naphthalenediol were of 'scintillation' grade. Lyophilized luciferase of *Photobacterium leiognathi* bacteria was provided by the Biotechnology Section of the Institute of Biophysics, Russian Academy of Sciences, Siberian Branch (17).

Equipment

Emission spectra were recorded by Fluorolog-3.22 (SPEX Industries, USA) and automatically corrected for the characteristics of this instrument. Final fluorescence spectra were derived by subtraction of the solvent spectrum from the sample spectrum.

Time-resolved fluorescence measurements were carried out using mode-locked continuous-wave lasers for excitation and a time-correlated photon-counting detection technique, as described previously (18). The repetition rate of excitation pulses was 951.2 kHz, the excitation wavelengths were 300 nm (for the luciferase samples) and 343 nm (for all other samples), the duration about 4 ps full-width at half-maximum. The samples were in 0.5 ml and 10 mm light path fused silica cuvettes, placed in a temperature-controlled (20°C) sample holder. Extreme care was taken to avoid artifacts from depolarization effects [see details in (19, 21)]. Detection electronics were time-correlated single-photon counting modules, described elsewhere (20). To select the detection wavelengths, interference filters were used (Schott 348.8 and 426.1 nm). All possible instrumental sources for distortion of data were minimized to below the noise level of normal photon statistics (21). Measurements consisted of repeated sequences of measuring during 10 s parallel and 10 s perpendicular polarized emission. The number of sequences was chosen to yield a peak content in the data files of up to 100 000 counts. After measuring the fluorescence of the sample, the background emission of the solvent was measured and used for background subtraction. The solvents applied were ethanol of fluorescence spectroscopy grade or Nanopure™ water, and also tested for artificial luminescence. For obtaining a dynamic instrumental response of the experimental set-up, the single exponential fluorescence decay of paraterphenyl was measured in a mixture of cyclohexane and CCl_4 (for experiments with excitation at 300 nm) and POPOP in an ethanol solution (for the other experiments).

Data analysis

Data processing was performed using a home-built computer program [22]. Anisotropy decay curves were modelled as a sum of exponentials. The quality of the fit was judged from three parameters: function of weighted residuals; autocorrelation function; and χ^2 parameter. For all the results presented in this work, the two former parameters were randomly distributed around zero, and χ^2 were minimized to 0.9–1.2.

RESULTS

The fluorescence anisotropy decay of four aromatic compounds was studied in different media. Time-resolved fluorescence anisotropy of anthracene, POPOP, MSB and 1,4-naphthalenediol was measured in: (a) ethanol; (b) water–ethanol solutions; and (c) water–ethanol solutions in the presence of bacterial luciferase. The water–ethanol solutions were prepared by mixing the ethanol solutions of the substances with water at the ratio of 1:100. This ratio was chosen to minimize the ethanol influence on the enzyme and to design media conditions close to optimal for luciferase functioning.

Fluorescence spectra of the aromatic compounds were

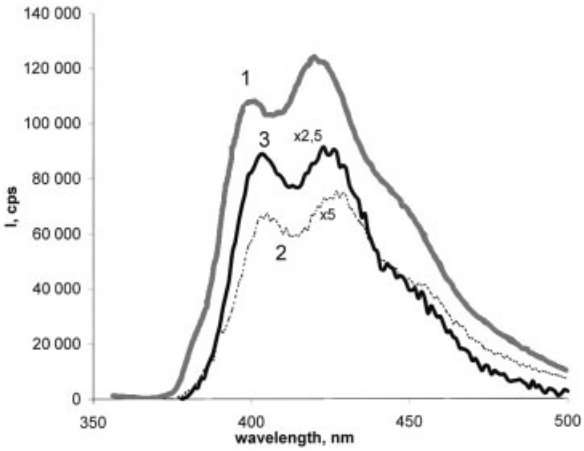

Figure 1. Fluorescence spectra of MSB, $C = 10^{-7}$ mol/L, excitation 343 nm, t = 20°C: 1, ethanol solution; 2, water–ethanol solution (100:1); 3, water–ethanol solution (100:1) with *P. lieognathi* luciferase (2×10^{-6} mol/L).

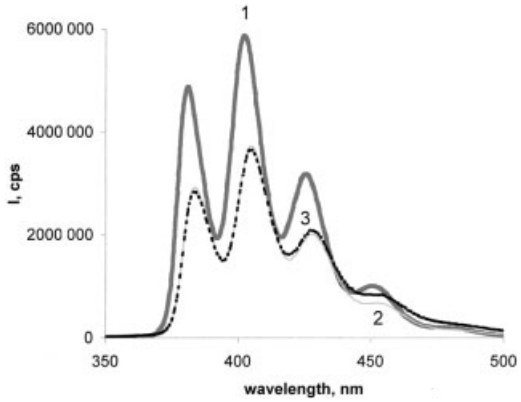

Figure 3. Fluorescence spectra of anthracene, $C = 2 \times 10^{-6}$ mol/L, excitation 343 nm, t = 20°C: 1, ethanol solution; 2, water–ethanol solution (100:1); 3, water–ethanol solution (100:1) with *P. lieognathi* luciferase (1.4×10^{-5} mol/L). Spectra 2 and 3 are multiplied to 8.

recorded at excitation of 343 nm, corresponding to the absorption region of the compounds. The results are shown in Figs 1–4. All molecules were found to exhibit a bathochromic shift of their spectra in more polar water–ethanol medium (cf. curves 1 and 2, Figs 1–4). The most pronounced shift was registered for POPOP (40 nm; Fig. 2). The weakest influence of the medium on the spectrum shape was recorded for the anthracene molecules (Fig. 3). The spectral maxima of the compounds in the presence of luciferase were found between those in the ethanol and water–ethanol solutions (Figs 1, 2, 4).

Another considerable effect of the media is that compounds' fluorescence is about one order weaker in the water–ethanol solution than in ethanol (Figs 1–4). Addition of luciferase enhances the fluorescence intensity of MSB, POPOP (Figs 1 and 2). The presence of the enzyme has no substantial influence on the fluorescent intensity of anthracene and 1,4-naphthalenediol (Figs 3 and 4).

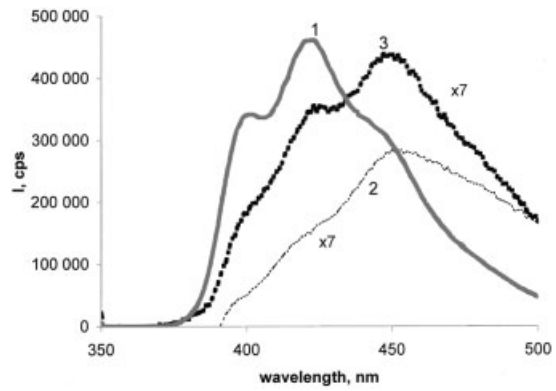

Figure 2. Fluorescence spectra of POPOP, $C = 10^{-7}$ mol/L, excitation 343 nm, t = 20°C: 1, ethanol solution; 2, water–ethanol solution (100:1); 3, water–ethanol solution (100:1) with *P. lieognathi* luciferase (2×10^{-6} mol/L).

These water–ethanol solutions exhibited instability after preparation: the intensity of their fluorescence slowly decreased with time (data not shown). Because of this, we used only fresh water–ethanol solutions in time-resolved experiments.

The typical fluorescence anisotropy decay curves obtained in different media are shown in Fig. 5. MSB was chosen as an example. It is apparent that in the water–ethanol solution anisotropy decay of MSB becomes substantially prolonged. Addition of luciferase into the water–ethanol solution accelerates anisotropy decay. Similar regularities were observed for anisotropy of the other compounds.

In Table 1 the results of the anisotropy data analysis are summarized. It is seen that in the ethanol solutions the anisotropy decays show single-exponential behaviour with short (0.15–0.29 ns) rotational correlation times.

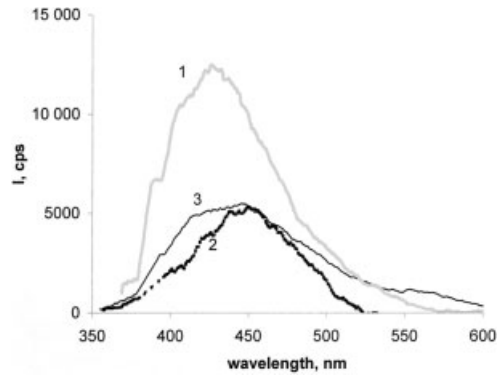

Figure 4. Fluorescence spectra of 1.4-naphthalendiol, $C = 4 \times 10^{-7}$ mol/L, excitation 343 nm, t = 20°C: 1, ethanol solution; 2, water–ethanol solution (100:1); 3, water–ethanol solution (100:1) with *P. lieognathi* luciferase (1.4×10^{-6} mol/L).

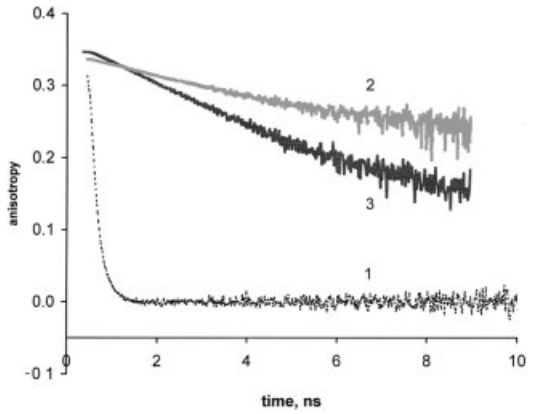

Figure 5. Fluorescence anisotropy decay curves of MSB in ethanol (1), water–ethanol (100:1) solution (2), and in water–ethanol (100:1) solution in the presence of *P. leiognathi* luciferase (3). Excitation 343 nm, detection 426 nm, t = 20°C. Parameters of the fitted curves are presented in Table 1.

Only anthracene has the second long-lived component of low intensity. In contrast, the average rotational correlation time of fluorophores in a more polar medium (water–ethanol) is several orders higher (1.2–32 ns). The intervals for ϕ-values (in parentheses) are wide in this case. Fitting of the anisotropy decay in water–ethanol anthracene solution with the exponential model appeared to be impossible, due to very low intensity of its luminescence. In the presence of luciferase, the rotational correlation times were in the range 4–12 ns (Table 1). To define the dynamic properties of the luciferase macromolecule, we measured the fluorescence anisotropy of its aqueous solution (excitation 300 nm and detection 340 nm correspond the absorption and fluorescence peaks of tryptophan residuals, respectively). We found that the anisotropy decay of luciferase can be fitted by a biexponential function with the rotational correlation time of the main component of 34 ns (Table 1) and initial anisotropy of about 0.22. It should be noted that for a spherical protein with mass 77 kDa in an aqueous medium at 20°C, the rotational correlation time is 28 ns (Stokes–Einstein equation was used (16)). The longer time obtained in our experiments is probably due to deviation of the shape of the luciferase macromolecule from spherical (it is known that for *V. harvei* luciferase the axial ratio is about 1.5 (23); probably, this is approximately the same for *P. leiognathi* luciferase used in this work). The short component of luciferase's anisotropy decay ($\varphi_1 = 0.44$; Table 1) suggests the relative mobility of some tryptophan residues responsible for fluorescence of this protein in these experimental conditions.

DISCUSSION

The data obtained reveal the behavioural peculiarities of aromatic hydrophobic molecules in different media.

Red shift of the fluorescence spectra with increasing polarity of the solvent is typical for compounds characterized by radiational transfers of the $S_{\pi\pi}$*–S_o type (15). As a rule, such shifts do not exceed a few nanometers. We probably observed the clear effect of medium polarity for the MSB and anthracene cases (Figs 1 and 3, curves 1 and 2). The larger shifts of the emission maxima that appeared to be shown for POPOP and 1.4-naphthalenediol samples (40 and 20 nm, respectively; Figs 2 and 4) might indicate the formation of the fluorescent aggregates of these compounds in water–

Table 1. Parameters of fluorescence anisotropy decay

Sample	β_1	φ_1 (ns)	β_2	φ_2 (ns)	$r(0)$
Ethanol solutions*					
1.4-Naphthalendiol	0.02	0.20 (0.12–0.30)	–	–	0.02
POPOP	0.30	0.29 (0.26–0.32)	–	–	0.30
MSB	0.34	0.15 (0.13–0.16)	–	–	0.34
Anthracene	0.20	0.21 (0.16–0.27)	0.02	7.4 (2.3–13.5)	0.22
Water–ethanol (100:1) solutions*					
1.4-Naphthalendiol	0.10	0.06 (0.03–0.11)	0.03	1.2 (0.8–2.0)	0.13
POPOP	0.11	0.93 (0.50–1.43)	0.19	33 (15–49)	0.30
MSB	0.34	23 (18–33)	–	–	0.34
Anthracene		Fitting appeared to be impossible			
Water–ethanol (100:1) solutions in the presence of bacterial luciferase (2×10^{-6} mol/L)*					
1.4-Naphthalendiol	0.06	0.40 (0.19–0.61)	0.14	11.4 (7.1–15.7)	0.20
POPOP	0.20	9.7 (7.7–12.6)	–	–	0.20
MSB	0.16	11.3 (9.1–14.5)	–	–	0.16
Anthracene	0.13	4.8 (3.7–7.7)	–	–	0.13
Water solution**					
Bacterial luciferase	0.04	0.44 (0.23–0.65)	0.22	34 (30–39)	0.26

* Conditions: 20°C, $C = 1 \times 10^{-7}$ mol/L, $\lambda_{exc} = 343$ nm, $\lambda_{det} = 426$ nm.
** Conditions: 20°C, $C = 10^{-6}$ mol/L, $\lambda_{exc} = 300$ nm, $\lambda_{det} = 340$ nm.
Errors for ϕ-values are presented in parentheses for 67% confidence intervals.

ethanol solutions [24]. The considerable quenching of fluorescence observed for all four compounds in water–ethanol medium (in comparison with the ethanol one) may implicate the formation of non-fluorescent molecular aggregates. The time-dependent decrease of emission intensity of water–ethanol solutions (data are not presented) indicates the gradual enlargement of particles in 'ageing' solutions followed by sedimentation (25).

Anisotropy decay parameters (Table 1) support the possibility of aggregation processes in more polar media. Ethanol solutions of compounds exhibit very fast anisotropy decay (Fig. 5; Table 1). This is specific for small molecules in true (molecular) solutions. In water–ethanol solutions, the fluorophores' average rotational correlation times are much higher (Table 1). Large errors for ϕ-values (in parentheses, Table 1) for POPOP and MSB in the water–ethanol media indicate a variety of sizes (mass distribution) of fluorescence aggregates.

Another conclusion we would like to make concerns the mechanism of interaction of bacterial luciferase with chemically inert substances used. On the one hand, the dynamics of fluorescent anisotropy of anthracene, POPOP, MSB and 1,4-naphthalenediol confirm the absence of specific binding of these molecules with bacterial luciferase. The rotational correlation times in water–ethanol solutions of these compounds in the presence of luciferase differ from φ-values specific for the enzyme (4–12 ns and 34 ns, respectively; Table 1). This indicates that no rigid (covalent) bonds between the examined molecules and the enzyme appeared. On the other hand, the presence of an excess of enzyme molecules (molar concentration ratio, 1:50) changes the luminescence characteristics of anthracene, POPOP, MSB and 1,4-naphthalenediol (Figs 1–4). Luciferase partially 'removes' the influence of water molecules on the spectral properties of these hydrophobic molecules. This is manifested by both blue shift of the emission spectra and increase of the fluorescence intensity of the water–ethanol solutions as a result of the addition of luciferase (Figs 1–3). These effects point to less polarity of the fluorophores' environment in solutions with luciferase than in water–ethanol solutions. The presence of the enzyme also changes the anisotropy decay parameters of the substances. It is evident from Table 1 that in the presence of luciferase φ-values do not coincide with φ, either in the ethanol or in the water–ethanol media (taking errors into account) and differs considerably from φ-values of the luciferase macromolecule. The effect of luciferase on the spectral properties of water–ethanol solutions can be explained by hydrophobic interaction between this enzyme and the molecules examined. The luciferase molecule is known to have several hydrophobic pockets (23). Anthracene, POPOP, MSB and 1.4-naphthalenediol molecules are apolar or slightly polar. The difference in ϕ-values of the fluorescent molecules in the presence and absence of luciferase indicates smaller average size of the light-emitting particles in the presence of luciferase. This fact might be explained by a possibility of adsorbing aromatic molecules by hydrophobic fragments of luciferase, changing the equilibrium of aggregate formation and preventing the following growth of the aggregates and their sedimentation. In addition, considerable differences between rotational correlation times of the molecules in solution with luciferase and those for luciferase itself suggests the relative mobility of the adsorbed molecules.

On the whole, we conclude that spectral data and anisotropy decay parameters gained for a number of fluorescent compounds suggest the possibility of hydrophobic interactions of these compounds with bacterial luciferase in solution. As we deal with uncharged and apolar (or slightly polar) particles, the main contribution into intermolecular interaction of the enzyme and aromatic molecule might be made by dispersion (or London) attractive forces. These forces can act considerably at a distance of < 1 nm. Such rapprochement of luciferase and aromatic molecules supports the feasibility of energy transfer by the exchange resonance mechanism from the bioluminescence emitter precursor onto the molecule available.

Thus, the results obtained show that the mechanism of sensitized luminescence of MSB, POPOP and anthracene in a bioluminescence system with their absorption spectra do not overlap with the bioluminescence spectrum. Intermolecular interaction of bacterial luciferase with aromatic compounds found in this work suggests the feasibility of energy transfer by the exchange resonance mechanism with a collision-interaction radius. The bacterial bioluminescence emitter precursor molecule can act as a donor and molecules of MSB, POPOP, anthracene and 1.4-naphthalenediol act as acceptors.

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