ANALYSIS OF THE RATIO OF QUANTUM YIELD AND FATTY ACID FORMATION OF *PHOTOBACTERIUM LEIOGNATHI* BIOLUMINESCENCE

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The ratio of quantum yield and fatty acid produced in a bioluminescent reaction catalyzed by the luciferase of *Photobacterium leiognathi* with tetradecanal and FMN chemically reduced by dithiotreitol in the absence and presence of organic solvents—acetone and dimethyl sulfoxide (DMSO) has been studied. In the absence of organic solvents the light per a molecule of produced myristic acid is 0.21 quantum. Addition of organic solvents the ratio of the light product and fatty acid shifts towards considerable increase of myristic acid production. With addition of 1–6 vol.% of DMSO and 0.15 vol.% of acetone the quantum yield was 0.13–0.07 and 0.04 quanta of light per a molecule of myristic acid, respectively.

Bacterial luciferase is a flavin monooxygenase catalyzing the oxidation reaction of long-chain aliphatic aldehyde (RCOH) to a respective fatty acid (RCOOH) with emission of light in the visible region

$$FMNH_2 + O_2 + RCHO - RCOOH + FMN + H_2O + h\nu.$$

All bacterial luciferases exhibit bioluminescent activity with aldehydes having the length of chain from 8 to 16 carbon atoms. Maximum luminescence intensity, the total quantum yield, light emission decay constant are determined by the luciferase type and length of chain of the aliphatic aldehyde [1]. The total quantum yield of the bioluminescent reaction is defined as the quantity of light quanta emitted in the course of a catalytic act. Actually, the quantum yield can be calculated as the ratio of the number of quanta released in the course of reaction to the number of molecules of each substrate processed in the course of reaction; or to the number of enzyme turnovers; or to the number of product molecules produced [2].

Oxidation of aldehyde to a respective fatty acid has been proved experimentally. Meanwhile, the evidence about the ratio of the fatty acid forming in the course of reaction and the light quanta emitted is fragmentary which is probably due to the difficulties of evaluating experimentally microquantities of the fatty acid forming in the reaction. Only early works and for one substrate only which is not a natural substrate of the bacterial luciferase showed that the quantum yield of luminescence is not high [3, 4, 5]. Employment of radioactively labeled aldehyde made possible to demonstrate that for the luciferase of *Photobacterium phosphoreum* with decanal the quantum yield in terms of decanoic acid was 0.13 [3], for the luciferase of *Photobacterium fischeri* the quantum yield is in direct proportion to the amount of fatty acid forming in the course of reaction and the amount of light per a molecule of produced acid is 0.1 quantum [4].

This work examines the ratio of quantum yield and formation of fatty acid by the luciferase *Photobacterium leiognathi* with myristic aldehyde which is considered to be a natural substrate of bacterial bioluminescent reaction [1], and in varying microambience of the enzyme altered by addition of organic solvents into the reaction medium.

Materials and Methods

Purification of luciferase. The luciferase was isolated from the recombinant strain of *E. coli* carrying *lux A* and *B* genes of *P. leiognathi* luminous bacteria from the collection of the Institute of Biophysics (Russian Academy of Sciences, Siberian Branch). The enzyme was purified by ion-exchange chromatography on DEAE-sepharose and affinity chromatography on blue agarose by the methods described elsewhere [6]. The protein concentration was evaluated by microbiuret reaction [7]. The purity of luciferase preparations evaluated by electrophoresis in the polyacrylamid gel in the presence of DS-NA according to Lammly [8] was 90–95%.

Purification of aldehyde. Myristic aldehyde (C_{14}) (Merck, FRG) was purified by preparative thin-layer chromatography [9] on plates with Kieselgel type 60 (Merck). The separation was carried out in a chamber with a mixture of organic solvents hexane : ether : trichloroacetic acid at the ratio of 85%, 15%, and 1%, respectively. To identify the aldehyde location the plate edges were stained with rhodamine. The aldehyde was extracted with ether which was removed at a rotor evaporator. The purity of aldehyde was evaluated by thin-layer chromatography on silica gel. The sample was accelerated in the same mixture. The chromatogram was developed with 10% solution of H_2SO_4 on ethyl alcohol followed by heating. Purification produced a preparation with 90% purity. The aldehyde was purified immediately before experiments. FMN was obtained from Sigma (USA) and dithiothreitol (DTT) from Serva (FRG).

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Bioluminescent reaction conditions. The reaction was conducted with FMN chemically reduced in the presence of dithiothreitol. The reaction was initiated by addition of 100 μl of 0.68 mM FMN into the reaction mixture with 200 μl of luciferase at the initial concentration of 0.175 mM, 150 μl of 94 mM tetradecanal, 50 μl of 1 M DTT and 500 μl of 0.02 M phosphate buffer (pH 7.0). Under these conditions the luminescence is long, with continuous reduction of FMN the enzyme repeatedly reacts with the substrates. This allows the product to build up in amounts sufficient for analysis. The activity of luciferase was evaluated with a bioluminometer devised at the Institute of Biophysics (Russian Academy of Sciences, Siberian Branch). Kinetics was recorded with 2210 recorder (LKB-Wallac, Finland). The microambience of the enzyme was altered by addition of organic solvents, namely, acetone and dimethyl sulfoxide (DMSO), into the buffer solution.

Evaluation of the quantum yield and fatty acid produced in the bioluminescent reaction. After the enzyme was incubated for 40 min in the presence of substrates the reaction was interrupted by addition of chloroform into the reaction mixture and lipid components were extracted following a standard procedure [9] with following carrying out of methanolysis. For control use was made of the reaction mixture containing all components (including the enzyme) which were placed into chloroform without preliminary incubation. To take into account the fatty acid that can be produced by non-fermentative oxidation of aldehyde, the reaction mixture containing all components with the exception of the enzyme was incubated separately. Absolute amounts of myristic acid methyl ethers was determined by method of inner standard in the capacity which margaric acid (50 μl in each assay) was used. The inner standard was injected before extraction. Methyl ethers of the fatty acid was analysed with a chromatomass spectrometer GCD Plus from Hewlett Packard, equiping a column HP-5 ($L = 30$ m, $d = 0.25$ mm). The temperature (T) of assay injection 250 °C, T of detector 280 °C. The temperature program: initial $T = 100$ °C (2 min); the rate of temperature increase 8 °C/min to 170 °C; isotermic regime 3 min; the rate of temperature increase 10 °C/min to 230 °C; isotermic regime 10 min. Transpheric line temperature, 175 °C; ion source temperature, 200 °C; electron energy, 70 eV. Helium gas flow was kept at 1 ml/min. The amount of fatty acid was recalculated employing the Avogadro number and compared to the quantum yield of the reaction calculated as the area under the kinetic curve and expressed in quanta. The bioluminometer was calibrated by the luminol reaction [10].

Results and Discussion

With reaction running under the conditions when FMN is reduced in the presence of dithiothreitol the maximum intensity of luciferase luminescence is $(7.88 \pm 0.318) \times 10^{11}$ quanta$\cdot$s$^{-1}\cdot$mg^{-1} of enzyme. This values is approximately 4 orders less than in the reaction of the luciferase with photoreduced flavin. Yet, as the enzyme makes many turnovers and the light in the course of the first 20 min is emitted at constant intensity, the total quantity of quanta in the reaction in the buffer solution was $(1.19 \pm 0.038) \times 10^{15}$ in 40 min. During this period the reaction formed $(5.54 \pm 0.832) \times 10^{15}$ molecules of myristic acid. This amount was obtained after subtraction of the amount of fatty acid present on the luciferase and other reaction components. We should note that the fatty acid forming in non-fermentative oxidation of aldehyde in the course of 40 min does not exceed 10% of the background content of myristic acid in the used aldehyde preparation. Thus, under these conditions the light emitted is 0.21 quanta per molecule of myristic acid. However, it should be taken into account, that in repeated turnover of the enzyme the reaction can be inhibited by the product, and this can tell on the light yield of the reaction. This ratio cannot be considered absolute, either, because calibration of photomultipliers by luminol results in quantum yields of light-emitting reaction 2–2.7 times less than in calibration by radioactive standards [11].

Earlier our laboratory [12] obtained the effect of activation of bacterial bioluminescence in the presence of organic solvents (specifically, with addition of DMSO or acetone into the reaction medium) and initiation of reaction by photoreduced $FMNH_2$. Here we consider the effect of these solvents on the ratio of the quantum yield and production of the fatty acid under steady-state kinetics conditions with repeated turnovers of the enzyme and other methods of FMN reduction.

Performance of reaction in the presence of organic solvents in the reaction medium results in change both of the light emission intensity and of the quantum yield. So, the addition into the reaction medium of DMSO at the concentration of 1 vol.% lead to decrease of the light emission intensity and quantum yield by 12 and 16%, respectively, while at DMSO concentration of 6 vol.% the light emission intensity increased by 6% and quantum yield decreased by 10%. This way, we observe insignificant activation in bioluminescence. However, the amount of myristic acid produced in these experiments increased at higher

Table

Change of the maximu intensity (I_{max}) of luciferase luminescence, quantum yeild (Q) and fatty acid formation in absence and presence of organic solvents

	$I_{\max} \times 10^{11}$, q$\cdot$s^{-1}	Q (within 40 min) $\times 10^{15}$, quanta	Amount C_{14}-acid $\times 10^{15}$, molecules	Q/C_{14}-acid
Control	7.88 ± 0.318	1.19 ± 0.038	5.54 ± 0.832	0.21
1 vol.% DMSO	7 ± 0.312	1.00 ± 0.124	7.56 ± 2.106	0.13
6 vol.% DMSO	8.4 ± 0.636	1.08 ± 0.014	14.65 ± 5.89	0.07
0.15 vol.% aceton	6.15 ± 0.396	1.04 ± 0.084	25.52 ± 2.013	0.04

concentrations of DMSO in the reaction medium and was $(7.56\pm2.106)\times10^{15}$ and $(14.65\pm5.89)\times10^{15}$ molecules at 1 and 6 vol.% of DMSO, respectively. So, addition of DMSO into the reaction medium makes the ratio of the quantum yield and fatty acid production change and there is only 0.13 and 0.07 quanta of light produced per a molecule of fatty acid.

Addition into the reaction medium of acetone at the concentration of 0.15 vol.% even further decreases the light emission intensity and quantum yield, that drops by 22 and 13% as related to the control, respectively. Meanwhile production of the myristic acid was increased more than in addition of DMSO and was $(25.52\pm2.013)\times10^{15}$ molecules. The quantum yield on fatty molecule basis that under the said acetone concentrations was 0.04. The results obtained are summarized in Table.

From the results presented it is apparent that the bacterial bioluminescent reaction catalyzed by *P. leiognathi* under steady-state kinetics responds to small concentrations of added organic solvents. Decrease of the quantum yield of the reaction is attended by unproportional increase of fatty acid produced, so that the total quantity of emitted quanta of light and fatty acid molecules, like their ratio, varies with changing microambience of the enzyme. Neither does variation of the maximum reaction rate correlate with the variation of product yield and their ratio. For a number of proteins the recorded conformation reconstructions have been observed when the concentrations of organic solvents in reaction mixtures exceed 20–25% [13]. The observed disunity of production of light and fatty acid in the bioluminescent reaction does not seem to be due to the deformation of the enzyme in the presence of organic solvents. It is more probable that addition of solvents immediately affects the chemical stages of the fermentative reaction under study.

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