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O_2 INCORPORATION INTO A LONG-CHAIN FATTY ACID DURING BACTERIAL LUMINESCENCE

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The bioluminescence-dependent oxidation of a long-chain fatty aldehyde catalyzed by luciferase from *Photobacterium phosphoreum* has been studied in $^{18}O_2$ experiments. The results show the incorporation of one atom of molecular oxygen into the product, the corresponding fatty acid. This incorporation is not the result of exchange of $^{18}O_2$ with the aldehyde prior to oxidation to the acid, thereby indicating that the bacterial luciferase catalyzes an aldehyde monooxygenase reaction which is coupled with bioluminescence.

Introduction

The existing evidence indicates that the emission of light catalyzed by bacterial luciferase involves conversion of an aliphatic aldehyde (RCHO) with a carbon chain lenght longer than 6 to the corresponding acid (RCOOH) concomitant with the oxidation of $FMNH_2$ [1–3]. The overall reaction proceeds in several steps involving firstly binding of free $FMNH_2$ to luciferase (E) [4,5], and secondly, formation of an oxygenated intermediate. Added aldehyde is not required for these two steps but the resulting oxygenated enzyme · $FMNH_2$ finally emits light, reacting with the aldehyde as follows [6,7]:

$$E + FMNH_2 \rightarrow E \cdot FMNH_2 \xrightarrow{+O_2} E \cdot FMNH \cdot OOH$$

$$\xrightarrow{+RCHO} E + FMN + RCOOH + H_2O + h\nu$$

The question considered here concerns the possible incorporation of molecular oxygen into the product, fatty acid, during the process of bioluminescence. If the mechanism involves an oxygenase reaction to form the corresponding acid, then bacterial luciferase might be a monooxygenase similar to flavoprotein enzymes of the EC 1.14.13 category, in which oxidation of the substrate is directly coupled with interaction of molecular oxygen with a reduced flavin-enzyme complex [8]. Alternatively, it could be argued that the aldehyde might be oxidized to the acid by an oxidase mechanism. Although the latter explanation is less likely, since H_2O_2 is not the product formed by the luminescent process [7], an understanding of the fine details of the luciferase mechanism must still wait up on direct evidence to show whether molecular oxygen is incorporated into the fatty acid.

Materials and Methods

Chemicals. Bovine serum albumin and FMN were purchased from Sigma, NADH from Kyowa Hakko, lauraldehyde and lauric acid from Tokyo Kasei, and dithiothreitol and luminol from Nakarai Chemicals. Lauraldehyde was purified before use by thin-layer chromatography on silica gel plates (Merck 60F-524) in chloroform/diethyl ether

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(17 : 3, v/v) to preclude errors due to the possibly contaminated lauric acid. Luminol was further purified by recrystallization from acetic acid [9]. *N*-Methyl-*N*-nitrosourea was kindly provided by Dr. K. Inomata of our department. All other chemicals were of reagent grade.

Enzyme assay. The relative activity of luciferase was routinely assayed according to the method of Nakamura and Matsuda [10]. The peak intensity of luminescence was taken as a measure of initial rate. Total light emmitted was the integrated value of luminescence obtained during the period of 8 min. Absolute light intensity was measured by the method of Nakamura [9] with luminol as an internal standard with a Union Giken model RA-1100 stopped-flow spectrophotometer. For the FMN reductase assay, the reaction mixture contained in 1 ml of 100 mM potassium phosphate buffer (pH 7.0), 120 nmol NADH, 10 nmol FMN, 2 μmol dithiothreitol, and FMN reductase. The reaction was started at 20°C with the addition of NADH. One unit of the enzyme is defined as the number of μmol NADH oxidized per min.

Enzyme purification. The strain of *Photobacterium phosphoreum* was provided by Dr. T. Nakamura [10]. The ammonium sulfate fraction of luciferase was prepared from 120 g cells by the procedure described by Nakamura and Matsuda [10]. The luciferase was further purified by the following chromatography steps: DEAE-cellulose (5 × 30 cm column equilibrated with 33 mM potassium phosphate buffer, pH 7.0); DEAE-Sephadex A-50 (2.8 × 30 cm column equilibrated with the same buffer and eluted with a 600 ml gradient linear of phosphate buffer (pH 7.0) between 0.15 and 0.25 M); and Sephadex G-100 (5 × 50 cm column, equilibrated with 33 mM phophate buffer (pH 7.0) and filtered with the same buffer). The yield of the purified luciferase was 260 mg, corresponding to 10% recovery of activities from the crude extract. The purified enzyme had an absorption maximum at 278 nm and a shoulder at 375 nm. It showed a single band on polyacrylamide gel electrophoresis according to the method of Davis [11]. The specific activity of this preparation was $7.7 \cdot 10^{12}$ quanta $\cdot s^{-1} \cdot mg^{-1}$ protein at 20°C in the presence of 100 μM lauraldehyde.

The FMN reductase was purified from the crude luciferase extract by the following successive steps: fractionation with ammonium sulfate (0–0.5 saturation), dialysis against 33 mM potassium phosphate buffer (pH 7.0), containing 100 μM dithiothreitol and 1 mM EDTA, chromatography on DEAE-cellulose (equilibration with the same buffer, and elution with a similar buffer containing 100 mM phosphate), chromatography on a DEAE-Sephadex A-50 column (equilibration with 33 mM phosphate buffer (pH 7.0), containing 100 μM dithiothreitol and 1 mM EDTA, and elution with a gradient linear between phosphate concentrations of 70 and 110 mM in the same buffer), and gel filtration on a Sephadex G-75 column (equilibration and elution with 33 mM phosphate buffer (pH 7.0), containing 100 μM dithiothreitol and 1 mM EDTA). These procedures achieved a 740-fold purification which reduced 11 μmol FMN/min per mg protein under the standard assay conditions.

$^{18}O_2$ experiments. The luciferase reaction was carried out in Thunberg tubes. The standard reaction mixture contained, in the main tube, 20 nmol FMN, $5 \cdot 10^{-2}$ U FMN reductase and 20 nmol luciferase in 4.94 ml of 50 mM potassium phosphate buffer (pH 7.0), and in the side arm, 850 nmol NADH and 1.75 μmol lauraldehyde in 0.06 ml of the same buffer. After freezing in a solid CO_2/acetone bath, the frozen content was made anaerobic three times by alternate evacuation and equilibration with N_2 gas, and finally the N_2 was exchanged with a mixture of $^{18}O_2$ (25.1 atom% excess) and N_2 in a ratio of 1 : 4. The content was then allowed to stand in a thermostat at 25°C and the reaction was started by adding the contents of the side arm. After 8-min incubation, the reaction was terminated by quickly freezing the content in a solid CO_2/acetone bath and then 0.15 ml of 1 M NaOH was added. The unreacted lauraldehyde was extracted three times with 5 ml of diethyl ether each time. The combined solvent was evaporated to dryness. The pH of the water phase was then adjusted to pH 6.0 with 1 M HCl, and the lauric acid was extracted three times with 5 ml of diethyl ether each time. After the solvent was evaporated, the lauric acid was methylated by using *N*-nitroso-*N*-methylurea in diethyl ether [12].

Mass spectroscopic procedures. Each sample of lauraldehyde and methyl laurate obtained as de-

scribed above was dissolved in 40 μl acetone and analyzed on an instrument for combined gas chromatography-mass spectrometry (GC-MS) (a model JMS-D100 mass spectrometer with a model JGC-20KP gas-liquid chromatographic inlet, Nihon Denshi, Tokyo). The column (2 mm × 1 m, glass) was packed with Gaschrome Q (80–100 mesh) coated with Silicone OV-1 (1.5%). Helium gas flow was kept at 0.35 kg/cm^2; column temperatures, 126°C (lauraldehyde) and 140°C (methyl laurate); ion source temperature, 210°C; electron energy, 23 eV; accelerating voltage, 3 kV; and ionizing current, 300 μA. The ions, M, $M+2$, $M-127$, $(M+2)-127$, $M-140$, and $(M+2)-140$ in the mass spectra were used to calculate the incorporation of $^{18}O_2$ into methyl laurate. For lauraldehyde, fragment ion peaks of $M-140$ (CH_2CHOH) and $(M+2)-140$ were used.

Other methods. $^{18}O_2$ was prepared electrolytically from $H_2{}^{18}O$ (Yeda, 43.467 atom%) [13]. Protein was determined by a micro-modified biuret method [14] with crystalline bovine serum albumin as a standard.

Results

Interrelations of the luciferase-catalyzed lauric acid formation and bioluminescence are presented in Fig. 1. As shown here, the total light of the luminescence is directly proportional to the amount of formed lauric acid under the conditions employed in the present study.

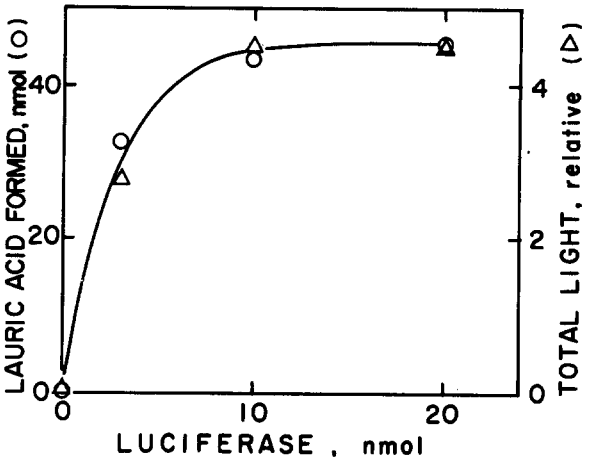

Fig. 1. Luciferase-catalyzed formation of lauric acid (○) and the total light emitted (△) from lauraldehyde. Experimental conditions were as described for $^{18}O_2$ experiments except that the reaction was carried out in regular test tubes in an atmosphere of air. The amount of lauric acid was analyzed with a Nihon Denshi model JGC-20KEP gas chromatograph. The column (4 mm×2 m) was packed with Chromosorb W(AW) (80–100 mesh), coated with poly(ethylene glycol succinate) (10%)/H_3PO_4 (1%). Column temperature was kept at 170°C for lauric acid and the gas flow was 2 kg/cm^2 (N_2), 1 kg/cm^2 (air) and 1 kg/cm^2 (H_2). The peak was identified by the retention time which was 9 min for lauric acid, and its amount was calculated using standard curve. In this figure, the formation of lauric acid is expressed after subtracting the nonenzymatic lauric acid formation from lauraldehyde which was omitted from the system.

When similar reactions were carried out in an atmosphere of $^{18}O_2/N_2$ (1 : 4), incorporation of atmospheric oxygen into lauric acid was found as

TABLE I

INCORPORATION OF $^{18}O_2$ INTO LAURIC ACID FORMED DURING LUCIFERASE REACTION

The figures in this table are calculated from subtracting the values of relative intensities of *a*, *M* and *M*+2; *b*, *M*−127 and (*M*+2)−127; and *c*, *M*−140 and (*M*+2)−140 in the air experiment from those, respectively, in the $^{18}O_2$ experiment after GC-MS analysis of methyl laurate prepared from the lauric acid formed during the incubation. Each analysis represents an average of at least six independent measurements.

System	atom% excess			^{18}O enrichment (%)		
	a	*b*	*c*	*a*	*b*	*c*
Complete	18±1	21±1	20±2	83±6[a]	96±7[a]	94± 9[a]
−Luciferase	0±1	1±1	2±2	0±6	6±4	10±11

[a] Calculated for incorporation of one atom of ^{18}O per molecule of product, after correction for dilution with lauric acid which was formed by the luciferase-independent oxidation of lauraldehyde. Under the conditions specified in these experiments, the value was 13% of the total lauric acid formed.

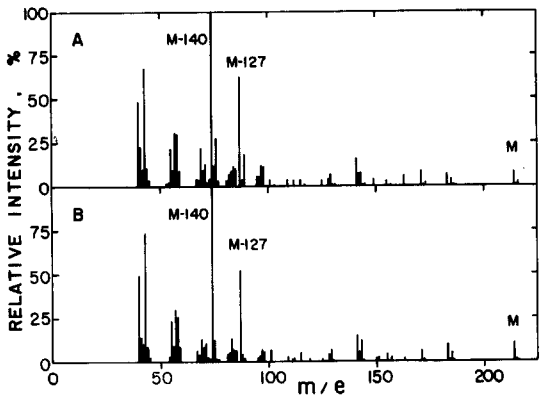

Fig. 2. Mass spectra of methyl laurate prepared from the lauric acid formed during luciferase reaction, either in an $^{18}O_2/N_2$ atmosphere (A) in which the O_2/N_2 ratio was 1:4 or air (B). *M*, $M-127$, and $M-140$ denote $CH_3(CH_2)_{10}COOCH_3$, $(CH_2)_2COOCH_3$, and $CH_2C(OH)OCH_3$, respectively.

evidenced by GC-MS analysis (Fig. 2). Shown in this figure are typical mass spectra of the methyl derivative of the lauric acid formed during the bacterial luciferase reaction, either with $^{18}O_2$ (Fig. 2A) or with air (Fig. 2B) as gas phase. The averaged data from similar determinations are summarized in Table I. The results in this table clearly show that the newly incorporated oxygen is located in the carboxyl group of the product. However, one of the major questions raised about these experimental results is the possibility that a direct exchange is taking place between lauraldehyde and $^{18}O_2$ prior to oxidation to the acid, either enzymatically or nonenzymatically. However, the mass spectrum of the unreacted lauraldehyde isolated from the same reaction mixture as used in the experiment in Fig. 2A indicates that the oxygen atom of the aldehyde has not been exchanged with atmospheric O_2 (not shown in Table I). These results lead to the conclusion that the bacterial luciferase catalyzes incorporation of molecular oxygen which is coupled to the bioluminescence.

Discussion

The results presented here have demonstrated the bioluminescence-coupled incorporation of molecular oxygen into the oxidation product of lauraldehyde, lauric acid. These results are consistent with the proposed mechanism for bacterial luciferase reaction in which the enzyme · FMNH · OOH reacts with an aldehyde, leading to bioluminescence and the formation of the corresponding acid [1–7].

In this regard, bacterial luciferase is analogous to flavoprotein hydroxylases as exemplified by salicylate hydroxylase (EC 1.14.13.1), in which the reduced form, i.e., the complex between the enzyme-salicylate and fully reduced flavin, reacts with O_2 to form hydroxylated product, catechol [8,15]. A difference is that the hydroxylases do not emit light. Our preliminary examination indicated that no light was emitted by 10^7-times greater amounts of salicylate hydroxylase in terms of electron turnover number than the minimum detectable amounts of the light-emitting luciferase.

Our present results are also consistent with the conception that in many other luciferase reactions found in animals such as *Cypridina* [16,17], *Renilla* [18], and the firefly [17,19], the bioluminescence reactions are coupled with oxygenation of the respective luciferins. However, it should be pointed out here that aldehyde oxidation by an oxygenase mechanism, such as that shown in this report, is unique. To our knowledge, the only example of an aldehyde oxygenase reaction so far reported is the 2-oxoglutarate-coupled conversion of 5-formyluracil to 5-carboxyluracil catalyzed by a *Neurospora crassa* enzyme (EC 1.14.11.6) [20]. In contrast, in many biological reactions an aldehyde is oxidized to the corresponding acid by either an oxidase or dehydrogenase mechanism, and hence, the incorporated oxygen should be derived from the water molecule.

Acknowledgements

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