Reactions Involved in Bioluminescence Systems of Limpet (*Latia neritoides*) and Luminous Bacteria

(*Achromobacter fischeri*/aldehyde/luciferin/luciferase/flavin/quantum yield)

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Communicated by Henry Eyring, May 30, 1972

ABSTRACT Luminescence in *Latia* involves a specific flavoprotein enzyme ("luciferase"), which has a tightly bound flavin group constituting the light-emitter. The overall reaction includes oxidation of a specific substrate ("luciferin," an enol formate derivative of an aliphatic aldehyde), by 2 O₂ molecules, in the presence of a "purple protein" cofactor, yielding a ketone, HCOOH, CO₂, and light. In *Achromobacter*, a required aliphatic aldehyde, which is functionally equivalent to *Latia* luciferin, is oxidized to an acid containing the same hydrocarbon chain as the aldehyde; this reaction proceeds in the presence of bacterial luciferase and reduced flavin mononucleotide with a quantum yield of 0.17 ± 0.1 photons per aldehyde molecule that is independent of aldehyde chain length from 9 to at least 14 carbons.

Although limpets and bacteria are only very distantly related in an evolutionary sense, some basic similarities are evident in the biochemistry of their luminescence systems: (a) the requirement for a colorless, hydrophobic enol-formate of an aliphatic aldehyde in *Latia* (1), and, similarly, a colorless, hydrophobic, aliphatic aldehyde in bacteria (2); (b) the long-known requirement for a flavin in bacteria (2) and, according to more recent evidence, probably also in *Latia*—as judged by correspondence between the spectral distribution of the bioluminescence reaction (ref. 1, Fig. 4) and the fluorescence emission of flavin. Moreover, the possibility of an oxidative splitting of a C-C bond of the aldehyde in luminescence of the bacterial system seems reasonable since splitting of C-C bonds occurs not only in *Latia* (1), but also in firefly (3, 4) and *Cypridina* (5, 6). If such splitting of a C-C bond does occur in the bacterial system, the possibility suggests itself that the product would very likely be an aldehyde that would be susceptible to a sequential reaction of the same sort, ultimately resulting in a chain length of 7 or less carbons, too short an aldehyde (see below) to be effective in luminescence.

The existing evidence indicates that the bioluminescence reaction of *Latia* proceeds as follows (1):

The stoichiometry of the O_2 and the $HCOOH$, as well as the identity of the light-emitting molecule or complex, have not been established.

The reactions involved in extracts of luminous bacteria may be diagrammed as follows; the minimal requirements are represented by the second equation, and sustained luminescence by both equations (2):

The emitter in this system, according to recent evidence (7), is the flavin mononucleotide cation, *i.e.*, FMNH⁺. The role of R-CHO, representing any of a homologous series of saturated, long-chain aliphatic aldehydes of more than 7 carbons, has remained unestablished. Two chief interpretations have been suggested, either that it furnishes part of the energy for light emission by oxidation of the terminal aldehyde to a carboxyl group (8, 9), or that it promotes a reaction of high quantum yield by causing a favorable conformation of luciferase (10).

MATERIALS AND METHODS

Latia luciferase and purple protein were extracted and purified as reported (1). Synthetic *Latia* luciferin (11, 12) was used. Long-chain saturated aldehydes (purity 95–98%) were fractionally distilled, and immediately dissolved to make 0.03 M solutions in methanol. FMNH₂ was prepared by reduction of FMN in water with platinized asbestos and hydrogen.

Light emission was measured by a Hamamatsu R-136 photomultiplier apparatus and recorded as total (integrated) photons against time. Calibrations and corrections to express the data in absolute units of photons were made on the combined basis of (a), the response of the photomultiplier to quanta yielded in the *Cypridina* luciferin-luciferase reaction (13), (b) the calibrated sensitivity of this photomultiplier tube to different wavelengths of light, and (c) the spectral distribution of intensity of light emitted in the isolated systems from *Cypridina* ($\lambda_{\text{max}} = 465 \text{ nm}$), *A. fischeri* ($\lambda_{\text{max}} = 489 \text{ nm}$), and *Latia* ($\lambda_{\text{max}} = 536 \text{ nm}$).

For mass spectrometry, specimens of the reaction products of bioluminescence of *Latia* luciferin were prepared as follows. Phosphate buffer (0.01 M, pH 6.8) containing 1.2 mM ascorbic acid, was first bubbled with H₂. 15 ml of this buffer plus 600 µg of luciferin in 0.22 ml ethanol were placed in one of the Y-arms of a specially made Y-shaped vessel, and a mixture of 35 ml of the buffer containing 1 ml of *Latia* luciferase ($A_{280} = 4.2$), 25 µl of purple protein ($A_{280} = 5.6$), and 20 mg of DPNH were placed in the other arm. The vessel was frozen in a dry ice-acetone bath and evacuated 15 min by a vacuum pump. After introduction of ¹⁸O₂ (99%), the contents were thawed

and the luminescence reaction was started by mixture of the two solutions at 10°. The resultant light ceased after 25 min. The vessel was then frozen again, and CO₂ for mass spectrometry was collected as described (6). The remaining frozen solution in the vessel was thawed and extracted three times with hexane; the combined hexane extracts were then evaporated for mass spectrometry.

Formic acid in the product of *Latia* luminescence was colorimetrically determined as follows: to 4.7 ml of a solution of 0.05 M sodium acetate (pH 6.3) containing 1 mM ascorbic acid, plus 0.1 ml of luciferase solution ($A_{280} = 1.1$), 10 μ l of protein solution ($A_{280} = 1.4$), and 2 mg of DPNH, was added 54 μ g of luciferin in 20 μ l of ethanol. When the resultant luminescence ceased, 0.4 ml of concentrated HCl was added, and 2.5 ml of the mixture was treated with a strip of magnesium to reduce formic acid to formaldehyde. Chromotropic acid (0.1 ml of 2% aqueous solution) was added to 2 ml of the reduction product, then this mixture was slowly mixed with 3 ml of concentrated H₂SO₄. After 1 hr, absorbance was measured at 580 nm against a similarly treated control that lacked luciferin. Absence of formaldehyde before the reduction was confirmed with the same color test.

The activity of bacterial luciferase preparations was measured by the initial maximum rate of light production after rapid injection of 0.5 ml of 60 μ M FMNH₂ into 0.85 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.1% bovine serum albumin, 5 mM 2-mercaptoethanol, 0.135 μ mol of decanal or dodecanal, and the specimen of luciferase.

The method for purification of bacterial luciferase was based on those of Hastings and associates (14, 15) and Kuwabara *et al.* (16), with some modifications in an effort to obtain highly purified luciferase along with adequate FMN reductase activity. A paste of centrifuged cells of *A. fischeri* was stirred into 20 volumes of cold water. After 30 min, the mixture was centrifuged; luciferase in the supernatant was adsorbed on DEAE-cellulose by the batch method, and was subsequently eluted with 0.45 M NaCl. After this step, 1 mM 2-mercaptoethanol was included in all buffer solutions. The eluted luciferase was fractionated with (NH₄)₂SO₄; the precipitate between 0.4 and 0.75 saturation was saved. It was then purified by two successive column chromatography steps on DEAE-cellulose, with elution by a linear gradient of NaCl concentration from 0.1 to 0.6 M. The best fraction was further purified by repeated refractionation by (NH₄)₂SO₄; the fraction insoluble between 0.55 and 0.65 saturation was saved. The product showed good FMN reductase activity, together with luciferase purified to about 55% of homogeneity (activity: 3.5×10^{13} and 5.5×10^{10} photons sec⁻¹ml⁻¹ at 25° for a solution of $A_{280} = 1.0$, with decanal and without added aldehyde, respectively). This preparation was used in most of the experiments on bacterial luminescence described in this paper. The same preparation easily yielded practically pure luciferase after chromatography on Sephadex G-100, followed by a final fractionation with (NH₄)₂SO₄. This product revealed (a) only a single prominent band, without any other detectable bands upon polyacrylamide-gel electrophoresis, (b) almost negligible FMN reductase activity, and (c) luciferase activity ($A_{280} = 1.0$) of 6.2×10^{13} photons sec⁻¹ml⁻¹ at 25° with decanal, or 1.4×10^{14} photons with dodecanal. The absorption spectrum revealed, in addition to the 280-nm protein peak ($A_{280}/A_{260} = 1.84$), only a very low shoulder ($A_{300}/A_{280} = 0.02$).

Samples for mass spectrometry of the product of bacterial luminescence were prepared as follows. The reaction was initiated by addition of 2 ml of 60 μ M FMN and 0.3 ml of 1% DPNH to a mixture of 3 ml of 0.05 M phosphate buffer (pH 7.0) containing 0.5 mM 2-mercaptoethanol plus luciferase (activity = 1.2×10^{14} photons sec⁻¹ with decanal) and 0.3 μ mol of tetradecanal or dodecanal. Luminescence ceased within 5–10 min. The solution was acidified with three drops of concentrated H₂SO₄ and extracted three times with ether-hexane 2:1. The extracts were combined, washed with water, dried with anhydrous Na₂SO₄, and concentrated for mass spectrometry.

RESULTS AND DISCUSSION

With *Latia*, both the intensity of luminescence and the quantum yield are increased by prior addition of 1 mM ascorbate, or somewhat less increased by an optimum concentration of DPNH (1). DPNH and ascorbate together caused an especially great increase; the quantum yield was 0.003 einsteins per mole of luciferin at 25° with ascorbate alone, but was 0.009 with ascorbate plus optimum DPNH. A small amount of sodium dithionite, added to luciferase plus purple protein before the addition of luciferin, caused almost as strong an activation as DPNH plus ascorbate.

The amount of formic acid in the *Latia* reaction mixtures at the end of a luminescence reaction was the same as that found when the reaction mixture contained no active luciferase, but instead contained acid-denatured luciferase, i.e., mixtures that yielded no luminescence. In both cases, 1 mol of luciferin gave 1 mol of formic acid—with acid-denatured luciferase by an easy hydrolysis. Mass spectrometry revealed that when the luminescence reaction was performed with ¹⁸O₂, compound II (see below) labeled with ¹⁸O (more than 60%), as well as CO₂, was produced. However, no C¹⁸O₂ was found and, unfortunately, C¹⁸O¹⁶O could not be estimated because of the presence of ethanol. Thus, the overall reaction of *Latia* bioluminescence may be written:

in which XH₂ stands for a reducing agent or group. In accord with previous observations (1), as well as with the involvement of 2O₂, the above equation very likely embodies two sequential oxygenase reactions. The strong activation by suitable reducing agents is easily understandable by this equation. Moreover, it seems likely that one of the oxygenase reactions involves the formation of a dioxetane ring (see refs. 3–6) at the carbons of C=C—O (as an intermediate), followed by splitting of the C—C bond, whereas the other, seemingly mono-oxygenase reaction, results in oxidation of the =C—O carbon.

Seeking to identify the light-emitter in *Latia*, we studied fluorescence of the purple protein under various conditions of solvents and temperature. No condition was found wherein the fluorescence spectrum was similar to the bioluminescence spectrum. However, the normally colorless, nonfluorescent luciferase fluoresced visibly in alkaline solution. It fluoresced most strongly in KCN solution ($\lambda_{\text{max}} = 536$ nm), with an emission spectrum very similar to that of bioluminescence, and also to that of flavin, except for the 370-nm region of the excitation spectrum (Fig. 1). Thus, it seems highly probable that the light-emitter in *Latia* is a flavin group bound to the

FIG. 1. Corrected spectra, obtained from an Aminco-Bowman Spectrophotofluorometer, of *Latia* bioluminescence *in vitro*, and fluorescence of *Latia* luciferase ($A_{280} = 1.1$, in 0.1 M KCN) and of FAD (1.5 $\mu\text{g/ml}$ in 0.1 M KCN). Emission spectra were measured by excitation at 460 nm, and excitation spectra were measured at 535 nm. For the absorption spectrum, *Latia* luciferase ($A_{280} = 1.1$) was in phosphate buffer (pH 6.8).

luciferase, with a quenched fluorescence or in a reduced state; the binding of flavin to protein must be very tight, as it is in certain other enzymes such as succinate dehydrogenase. Efforts to split the chromophore from *Latia* luciferase by means of urea or acid failed.

In the bacterial system, for experiments on the quantitative relation between the amount of aldehyde added and the total light produced, it was convenient to use a luciferase preparation containing FMN reductase. We added DPNH plus FMN to a reaction mixture, resulting in a complete reaction of the aldehyde, as well as a fast production of light (within about 10 min), and minimal auto-oxidation of the aldehyde. The results for two aldehydes are illustrated in Fig. 2, which

FIG. 2. Relationship between total light emitted and the amount of added decanal (filled circles) or dodecanal (open circles) in the bacterial reaction. The luminescent reaction was initiated by the injection of 0.1 ml of 0.1% DPNH into a mixture of 0.5 ml of 60 μM FMN and 0.75 ml of 0.1 M phosphate buffer (pH 7.0), containing luciferase (activity: 1.2×10^{13} photons sec^{-1}), plus aldehyde in the amounts indicated (in 2–10 μl of methanol). The mixture also contained 0.1% bovine-serum albumin, and 5 mM 2-mercaptoethanol. The luminescent reactions, at 24–25°, were completed in about 10 min.

indicates a linear relation over a wide range of concentrations, up to at least 12 nmol/ml. Alteration of the amount of the luciferase, FMN, or DPNH several-fold did not affect the total light measured, except for slight differences due to the absorption of luminescence by FMN. Thus, there was no evidence of consumption of the aldehydes through unknown reactions in addition to the luminescence pathway.

The data of Table 1 indicate an almost constant quantum yield at 24–25° of 0.154 ± 0.1 einstein/mol of aldehyde of chain lengths of more than 8 up to at least 14 carbons. After allowance is made for light absorbed by FMN (computed from the decrease in apparent quantum yield at double the usual concentration of FMN), the corrected yield is 0.17 ± 0.1 .

The possible production of formic acid, and perhaps formaldehyde, in the bacterial system was investigated by the same procedures used for the *Latia* system, except that phosphate buffer was replaced by 0.01 M Tris·HCl. Neither HCOOH nor HCHO was found.

In an effort to detect CO_2 as a possible product in the bacterial system by mass spectrometry, the luminescence reaction was carried out on a scale 10 times that of the experiments of Fig. 2. Dodecanal (1.5 μmol), luciferase (1.2×10^{14} photons sec^{-1}), and $^{18}\text{O}_2$ (99%) were used, and the CO_2 was collected as described for *Latia*. Some CO_2 was found. However, this CO_2 is of questionable significance in the luminescence reactions because the content of ^{18}O in the CO_2 was only about 1.5 times that of the 0.2% in its natural abundance, and because the amount of CO_2 found was much less than would be expected from 1.5 μmol of aldehyde.

The foregoing data indicate that a suitable aldehyde is consumed in bacterial luminescence. It seems likely that the aldehyde is oxidized at or near the terminal aldehyde group, as suggested by previous data (17), leaving the rest of the hydrocarbon chain intact in a form that should be extractable with an appropriate organic solvent. Mass spectra of ether-hexane extracts of acidified products of the luminescence reaction with tetradecanal (Fig. 3) and dodecanal were almost exactly the same as the mass spectra of pure tetradecanoic acid and dodecanoic acid, respectively, indicating that these

TABLE 1. Quantum yields with various aldehydes in the bacterial luminescence reaction at 24–25°

Aldehyde (1.5 nmol)	Carbons	Max. rate (tera- quanta per sec)	Total yield, tera- quanta	Quantum yield per molecule of aldehyde
Hexanal	6	0.02	5	0.002
Octanal	8	0.32	86	0.091
Nonanal	9	1.28	149	0.161
Decanal	10	0.78	143	0.155
Undecanal	11	0.80	134	0.144
Dodecanal	12	3.63	139	0.150
Tetradecanal	14	5.00	152	0.164
Not added	—	0.01	3	—

Reaction conditions were as described for Fig. 2. Quantum yields with aldehydes are corrected for the slight luminescence of controls without added aldehyde. The luminescent reaction was largely complete in about 5 min, and essentially fully complete in 15 min. Incubation of the reaction mixture for 5 min before addition of DPNH had no effect on quantum yield, but the yield was reduced by 5% when FMN alone was added after 5 min of incubation of the other components.

FIG. 3. Mass spectrum (70 eV) of an ether-hexane extract of the product of the bacterial luminescence reaction run with tetradecanal. Minor peaks at m/e 206 and 236, as well as moderately strong peaks at m/e 91 and 149, were shown by control experiments to be due to contaminants in the solvents used, especially the water.

acids were indeed the products. Moreover, the yield of these acids obtained after oxidation of the aldehydes was at least roughly quantitative, as shown by control experiments wherein the acids, instead of the aldehydes, were added to the reaction mixture, which was then extracted and analyzed in the same manner. Oxidation of R-CHO to R-COOH yields some 70 Cal, which, together with oxidation of FMNH₂, more than meets the energy requirements of the light emitted.

Finally, the possibility that a reaction other than that of aldehyde directly to acid might be involved can be discounted on the grounds that, even if such a different reaction had a quantum yield of 1, it would have to yield a product in excess of 17 mole-percent of the acids, and no such products were found by mass spectrometry. Thus, the following overall reaction seems justified (see refs 8, 19):

A possible mechanism for this reaction, on the assumption that "intermediate II" of Hastings and Gibson (18) contains a hydroperoxide group (—OOH), would be addition of the hydroperoxide to the carbonyl group of the aldehyde, followed by splitting of the O—O bond to give a carboxylic acid (20).

Since the aldehyde in bacterial luminescence has the same function as the luciferin of *Latia*, the term luciferin seems equally applicable in both systems, even though present knowledge (see ref. 21) makes this term only a convenient anachronism. In any event, an aspect of general interest is that luminescence of both systems involves the oxidation of a colorless, aliphatic substrate by a flavin enzyme, with the flavin as the light-emitter. Moreover, in *Latia* it seems very likely that the second step of the two reactions with O₂ more directly pertains to light-emission, and that it represents a mono-oxygenase reaction, as in the bacterial system. Finally, no luminescence system other than these two is known that consists of the same chemical and functional relations between oxidizable substrate, enzyme, and light-emitting molecule.

For a supply of synthetic luciferin of the *Latia* system, we are indebted to Dr. Y. Kishi of Nagoya University, Japan. We

thank the National Science Foundation and the Office of Naval Research for support, in part, of this research.

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