The Effect of Flavin Isomers and Analogues upon the Color of Bacterial Bioluminescence*

GEORGE MITCHELL‡ AND J. WOODLAND HASTINGS
From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

SUMMARY

Bioluminescence centered at about 490 nm occurs in the bacterial luciferase system upon the oxidation of reduced flavin mononucleotide by molecular oxygen. Although excited FMN is the apparent candidate for the emitting molecule, the color of its fluorescence, which is centered at about 525 nm, does not correspond to that of the bioluminescence. In the experiments reported here flavins differing in their fluorescence emission were tested with bacterial luciferase. All were found to be active with the enzyme to a greater or lesser extent, and to result in a shift in the color of the emitted light. However, this shift did not parallel in any case the corresponding shift in the fluorescence emission of the compound. The results could not be readily explained in terms of solvent polarity effects. Although no specific molecular species can be designated as the excited state, it seems likely that it is a luciferase-bound complex and that it involves flavin.

Bacterial luciferase catalyzes a bioluminescent oxidation of reduced flavin mononucleotide in the presence of a long chain aldehyde (1). The over-all reaction may be represented as follows.

$$\text{FMNH}_2 + \text{O}_2 \xrightarrow{\text{luciferase}} \text{RCHO} \rightarrow \text{FMN} + \text{light}$$ (1)

It has not yet been possible to specify exactly either the stoichiometry and products of the reaction or the excited molecular species involved in the light emission. The experiments described here are concerned with the latter point.

When bacterial luciferase was first isolated and FMNH$_2$ was shown to be the substrate, it seemed logical to designate the excited singlet of FMN as the emitting species (2). But the fact that the fluorescence emission of FMN is centered at about 525 nm while the emission spectrum of bioluminescence is maximal at about 490 nm militated against this specific proposal (5). Two suggestions have been seriously entertained. First, the emission might derive from an enzyme-bound excited state of flavin modified in the bound state by environmental factors so as to give a blue shift (6). It is also possible that the flavin could be chemically altered in the course of the reaction via some covalent step, to give a species with the appropriate singlet level. Secondly, the emission might result from some other undetected enzyme-bound group or, indeed, from an aromatic amino acid residue of the luciferase itself (7, 8).

Neither of these alternatives has been easy either to exclude or to support. The fluorescence emission of iso-FMN (the 5,6-dimethyl isomer) is shifted to the red by about 20 nm. It was therefore tested as a substrate for luciferase with the expectation that, if active, the bioluminescence emission should be either similarly shifted or unchanged, supporting, respectively, the first or second of the suggestions mentioned above. It was discovered that bioluminescence with iso-FMNH$_2$ was indeed shifted, but to shorter wavelengths instead (Fig. 1).

A number of other flavins were therefore studied with regard to their activity with luciferase and the emission spectrum of the resulting bioluminescence. The results allow us to assert that while the molecular structure of the flavin can markedly influence the color of the bioluminescence, this effect cannot be directly correlated with the energy of the singlet-ground state transition level (e.g., fluorescence) of the molecule.

MATERIALS AND METHODS

The luciferase used in most of the experiments was isolated from a strain of luminous bacteria given the laboratory designation MAV. The origin and classification of this strain is unknown, but the properties of its luciferase are strikingly different from the "PF" luciferase from Photobacterium (Achromobacter) fischeri (ATCC 7744), the species used in previous studies reported from this laboratory (9). The luciferases from the two organisms differ in their specificities to the various flavins and in the color and kinetics of the resultant emission. Other differences between the luciferases will be described more fully elsewhere.

Luciferase was purified by modifications of the methods previously described (9), the details of which will be presented separately. The MAV luciferase was recrystallized twice to a constant specific activity while the PF enzyme, though not

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crystallized, was at least 95% pure as judged by acrylamide gel electrophoresis (10). The specific activities for luciferase differ, depending on the aldehyde used in the reaction (11), and are expressed in units of quanta sec⁻¹ per absorbance of 1 at 280 nm. For the MAV enzyme the specific activity with decanal was 2 × 10⁴ and with dodecanal 2.7 × 10⁴, while the values for the PF enzyme were 2.2 × 10⁴ and 6 × 10⁴, respectively. These numbers reveal the marked differences in the quantitative responses of the two luciferases to different aldehydes.

Luciferase was assayed as previously described (9) by rapidly mixing it with reduced flavin in the presence of dodecanal and oxygen. Reaction mixtures contained 0.02 ml of 1.25 M phosphate buffer (pH 7.0), 0.01 ml of a dodecanal suspension prepared by ultrasonic treatment of 10 μ of the aldehyde in 10 ml of distilled water, 0.2 ml of 1% bovine serum albumin, luciferase as specified, and water, to a volume of 1.5 ml. The reaction was then initiated by injecting from a syringe 1 ml of the flavin (about 5 × 10⁻⁴ M), reduced by bubbling hydrogen in the presence of platinumized asbestos. Reactions in which the spectral emission was determined were run at 9° in order to extend the light emission for a time sufficient to scan the spectrum (5). The luminescence of the MAV enzyme with dodecanal and FMNH₂ has a half-life of about 75 sec (k = 0.015 sec⁻¹) at 9°. Other reactions were run at 22° ± 2°.

Extracts of luminous bacteria contain a flavin reductase which is presumed to be the pathway in vivo for the reduction of flavin by DPNH (9, 12). The assay of the activity of the flavin analogues with this enzyme was carried out either by a spectrophotometric assay, measuring the disappearance of DPNH, or by coupling it to luciferase, with the use of the reaction mixture specified, with DPNH (2 × 10⁻⁴ M) and the various oxidized flavins added.

Luciferase was treated in 5 M guanidine HCl as described by Friedland and Hastings (10, 13). The protein is thereby dissociated into its subunits, and any noncovalently bound molecules are readily separated by dialysis or Sephadex chromatography carried out in the presence of 5 M guanidine HCl. The recovery of activity may be achieved by dilution, which presumably permits the peptide chains to refold. The protein was then concentrated by ultrafiltration (Amincon Corporation, Lexington, Massachusetts). In the experiments reported here approxi- mately 40% of the activity was recovered, although higher values, sometimes close to 100%, may be achieved (13).

Light was detected by the use of photomultiplier tubes calibrated with the standard of Hastings and Weber (14), amplified and recorded graphically. Bioluminescent emission spectra were determined with a motor-driven grating monochromator (Bausch and Lomb, 500 mm, 1200 lines per inch, blazed at 500 nm) in conjunction with two photomultipliers. One phototube served to monitor the bioluminescence at the entrance slit; the other recorded the light intensity at the exit slit as a function of wave length (15). The slits were set at 4 mm. Since we are primarily interested in spectral differences, the values given are not corrected for variations in phototube sensitivity and monochromator efficiency. The application of these corrections would shift the values to the red by 2 to 4 nm.

Absorption measurements of flavins were made in 0.05 M phosphate buffer at pH 7, with a Cary Model 15 spectrophotometer. Fluorescence measurements were made in the same solvent with the Aminco-Bowman spectrophotofluorometer modified by

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**RESULTS**

Although the absorption spectrum of iso-FMN is rather similar to that of FMN or riboflavin, its fluorescence has a lower quantum yield and is shifted to the red by about 20 nm (Fig. 1). Since the reduced iso-FMN was found to be weakly active with luciferase, it was of interest to see if the bioluminescence emission was similarly shifted. A substantial shift was indeed observed, but to shorter wave lengths instead. The data in Fig. 1 were used to provide ratio recording of the excitation intensity to the fluorescence intensity and fitted with gratings ruled at 1200 lines per inch. The values given are not corrected; the corrections required and their magnitude are similar to those mentioned above. Phosphorescence measurements were made with the standard Aminco-Bowman instrument (600 lines per inch gratings), with 50:50 propylene glycol-ethanol as the solvent.

Chemical reagents were of analytical quality where available. Commercial FMN and FAD were purified by DEAE-cellulose chromatography, as was 2-thio-FMN,¹ while the riboflavin was used without purification. Isoflavobin (Cälbochrom) was recrystallized prior to use. Iso-FMN, tetraacetylisoriboflavin, and lumiflavin 3-acetic acid were provided by Dr. W. Förö, while the other flavin analogues were a gift of Dr. W. Förö. Extinction coefficients were used to determine concentration (16, 17).

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¹ The starting material was quite impure and it was not shown that the chromatographic fraction used was definitely 2-thio-FMN. However, its absorption properties were close to those reported for that compound by Förö and Hemmerich (12). Our nomenclature for the flavins follows their usage.
were obtained and tested in the bioluminescent reaction. With 2-thio-FMN, another unusual result was obtained. Although the compound is nonfluorescent (and nonphosphorescent), both in water and in nonpolar solvents, its ability to stimulate bioluminescence is significant (Table I). This emission is red-shifted, as compared with FMN, peaking at about 534 nm (Fig. 2). It should be noted that the absorption of thio-FMN is also red-shifted by about 40 nm (Fig. 3).

Most of the other flavins tested are effective in stimulating bioluminescence to a greater or lesser degree (Table I). In the case of the 2-morpholino derivative, the intensity is relatively high, whereas the 2-(β-hydroxyethylimino) and the 2-phenyl-imino compounds, are respectively, 10- and 100-fold less intense. In these cases bioluminescence emission spectra are shifted to the region of 485 nm (Table I).

The fact that the color of the fluorescence emission of small molecules may be markedly shifted by binding to protein is usually attributed to environmental effects, for example, to a difference in the dielectric constant of the environment at the protein binding site (20-22). Although FMN does not bind to luciferase (9), the excited species produced in the chemiluminescence could very well be strongly bound to a protein site. The results described above might thus be accounted for in terms of the effect of the dielectric constant of the medium upon the emission, on the assumption that the emission shift is more pronounced with some molecules than with others. If such is the case, the effect of solvent polarity upon the fluorescence emission of iso-FMN should be more pronounced than upon that of FMN itself.

**Table I**

**Bioluminescence and fluorescence properties of various flavins**

Bioluminescence activity is expressed for the different flavins in percentage relative to that obtained with FMNH$_2$. Activities are given for the two luciferases (MAV and PF; see “Materials and Methods”); activities with DPNH were determined with a purified flavin reductase and crystalline guanidine-treated MAV luciferase, with the various flavins at a final concentration of 5 x 10$^{-5}$ M.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MAV Bioluminescence activity (%)</th>
<th>PF Bioluminescence activity (%)</th>
<th>$\lambda_{max}$</th>
<th>MAV $\epsilon_{max}$</th>
<th>PF $\epsilon_{max}$</th>
<th>MAV Bioluminescence activity with DPNH (%)</th>
<th>PF Bioluminescence activity with DPNH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMN</td>
<td>100.0</td>
<td>100.0</td>
<td>492</td>
<td>534</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td>1.6</td>
<td>0.7</td>
<td>464</td>
<td>524</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td>8.0</td>
<td>13.0</td>
<td>492</td>
<td>524</td>
<td>5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iso-FMN</td>
<td>3.5</td>
<td>14.0</td>
<td>472</td>
<td>543</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Thio-FMN</td>
<td>7.5</td>
<td>4.6</td>
<td>534</td>
<td>*</td>
<td>5.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Morpholino, 2-deoxy</td>
<td>11.5</td>
<td>18.5</td>
<td>484</td>
<td>536</td>
<td>0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-(β-Hydroxyethyl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>imino-FMN</td>
<td>1.6</td>
<td>2.5</td>
<td>486</td>
<td>525</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Acetyl-FMN</td>
<td>1.5</td>
<td>2.7</td>
<td>488</td>
<td>524</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Phenylimino-FMN</td>
<td>0.15</td>
<td>0.17</td>
<td>485</td>
<td>536</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoriboflavin</td>
<td>0.1</td>
<td>0.015</td>
<td>486</td>
<td>543</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetraacetylisoriboflavin</td>
<td>0.01</td>
<td>*</td>
<td>540</td>
<td>*</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumiflavin-3-acetic acid</td>
<td>0.5</td>
<td>0.1</td>
<td>*</td>
<td>*</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral red</td>
<td>0.32</td>
<td>0.15</td>
<td>485</td>
<td>*</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No added flavin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 2-Thio-FMN is nonfluorescent.

* These values were not determined.

Fluorescence emission spectra of three flavins were therefore measured in a number of different solvents and plotted according to the Kosower Z scale (23) of solvent polarity (Fig. 4). Although there is no special reason to suppose that the function should be linear, there is no strong indication from the results that the iso-FMN is more markedly affected by solvent polarity than is FMN.

These results prompted us to re-examine the enzyme preparation itself with regard to the possible occurrence of a species involved in the emission. We had previously observed that a number of different reducing agents (such as neutral red or dithionite) could stimulate a weak but appreciable bioluminescence. This observation was confirmed by Cormier and Kuwabara (24); it was later concluded that FMN was not involved in the emission because there was no flavin in the enzyme preparations used (25). These authors did not advance a suggestion for the emitter, however.
small but detectable quantities of an unidentified fluorescent material might be completely freed of this presumed contaminant by exhaustive dialysis against 5 M guanidine HCl. Although all measurable fluorescence was removed, the sensitivity of the bioluminescence measurement is greater and allowed us to see that a weak activity toward reduced neutral red and dithionite remained. The experiment was carried out with techniques previously described (10, 13), which have been shown to dissociate the luciferase subunits reversibly, presumably liberating all noncovalently bound material. Renaturation of luciferase in low ionic strength buffer occurs equally well with subunits dialyzed against 5 M guanidine HCl or with those isolated by Sephadex chromatography, thereby excluding all other species of lesser molecular weight.

Although the intensity is very low, we have determined the emission spectrum of the bioluminescence initiated by dithionite to be maximum at about 475 nm (with guanidine-treated enzyme). The neutral red emission (with the crystalline enzyme) peaks at about 485 nm. Since this activity is truly insignificant quantitatively in purified preparations, it should probably be excluded from serious consideration as regards the principal pathway and mechanism involved in flavin-initiated bioluminescence. This does not deny its inherent interest.

In crude extracts the reduction of FMN can be achieved by a soluble enzyme which utilizes reduced pyridine nucleotide.

\[
\text{DPNH + FMN} \xrightarrow{\text{reductase}} \text{DPN} + \text{FMNH}_2
\]

Bioluminescence is obtained by coupling this system with Reaction 1. This reductase has been isolated, purified, and studied in some detail by Duane,* who found (using a spectrophotometric assay) that it was inactive with iso-FMN. In the "coupled" light assay a slight stimulation was observed with iso-FMN, indicating that it actually is weakly active (Table I).

The other flavins were similarly tested in the coupled assay for their ability to stimulate bioluminescence. With the exception of 2-thio-FMN and riboflavin, which also stimulated slightly, no others exhibited activity. Several caused an actual inhibition of the "endogenous" level, i.e., the weak bioluminescence obtained with no added flavin. The reason for this has not been investigated.

**Table II**

Comparison of activities with different reductants of the MAV luciferase at different stages of purification.

<table>
<thead>
<tr>
<th>Preparation and specific activity</th>
<th>Neutral red</th>
<th>Dithionite</th>
<th>Riboflavin</th>
<th>2-Morpholino-2-deoxy FMN</th>
<th>2-(β-Hydroxyethyl)-FMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude (1.4 X 10^16) . . . . . . . .</td>
<td>2.0</td>
<td>1.1</td>
<td>5.5</td>
<td>10.0</td>
<td>3.4</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ ppt. (2.6 X 10^16) . . .</td>
<td>4.3</td>
<td>1.3</td>
<td>12.0</td>
<td>21.0</td>
<td>4.3</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography (7.8 X 10^16) . . . . . .</td>
<td>0.65</td>
<td>0.2</td>
<td>10.0</td>
<td>11.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Crystalline (2.0 X 10^16) . . . . . .</td>
<td>0.32</td>
<td>0.08</td>
<td>8.0</td>
<td>11.5</td>
<td>1.6</td>
</tr>
<tr>
<td>5 M Guanidine-Sephadex (8.5 X 10^16) . . . . . .</td>
<td>0.05</td>
<td>0.02</td>
<td>5.5</td>
<td>10.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**DISCUSSION**

While these experiments do not allow us to specify the electronically excited species responsible for the normal emission in bacterial bioluminescence, they do allow us to arrive at some useful conclusions.

In the normal reduced flavin-initiated bioluminescence we believe that the flavin molecule serves both as the reductant and as a participant in the excited state. What then is the nature and importance of the emission which occurs with nonflavin reductants, such as dithionite and neutral red? We believe that this emission is facilitated by some unidentified contaminating species which is bound to the enzyme, the involvement of the contaminant being confined to the second of the two postulated functions of flavin. The fact that the activity is appreciable in crude extracts but declines progressively during purification (Table II) supports this conclusion.

By contrast, the various flavin isomers and analogues appear to participate.

* W. Duane, unpublished results.
to possess genuine activity with luciferase, although in some instances it is very weak. The activity of these flavins does not seem to depend on the hypothetical contaminant, since they remain approximately the same relative activity with luciferase at various stages during its purification (Table II).

The conclusion that these flavins participate in the emission step is also supported by the observation that the flavin can affect both the duration of the bioluminescence and its color. It has been clearly shown (6) that the reaction of the reduced flavin together with the entry of the oxygen occurs during and is confined to the first fraction of a second after mixing, while the emission itself extends over tens of seconds thereafter, declining exponentially. The nature of the intermediate having this long lifetime, which ultimately gives rise to an excited species, is not known. But it is clear that since different flavins can have an effect on its lifetime, they are probably involved in some significant way. A similar argument, although not irrefutable, applies with regard to the color of the emitted light.

While these experiments strongly implicate flavin as a participant in the electronically excited complex which results in light emission, the excited species is apparently not the flavin singlet as such. The disparity between fluorescent and bioluminescent emission spectra does not appear to be explained by environmental effects. This disparity is even greater with most of the flavins tested in these experiments (notably iso-FMN), a further indication that some excited state other than the excited singlet is the species of interest. There is no reason to consider the triplet state of flavin in this connection, especially since its energy level is apparently invariant in the compounds examined.

The analogue 2-thio-FMN takes on special interest with regard to the nature of the excited state. It is neither fluorescent nor phosphorescent, yet its ability to stimulate bioluminescence is significant, and the light emitted is shifted to the red by more than 40 nm from that obtained with FMNH2.

In attempting to account for these results and to propose a candidate for the excited molecular species, we should again point out that the emission in bioluminescence derives from a state which is at a considerably higher energy level than is the singlet of flavin. One could entertain the idea that the initial excited state which is populated by the chemical reaction is an excited state of tryptophan. But unless the triplet were populated directly, the energy required would be almost prohibitive. Consideration has also been given to the idea that emission might occur via some kind of heteroexcimer (26), e.g. between flavin and some other species. But such a mechanism predicts that the wave length of the emission will be determined by the lowest available energy level in the complex, i.e. the flavin.

In spite of this difficulty, those ideas are of interest in connection with light-initiated bioluminescence (27, 28). In this case it was shown that light absorption (monophotonic) either by protein or by a species absorbing at about 400 nm could result in the emission of typical bioluminescence at about 490 nm. It has been recently shown by Mitchell (29) that this protein is separable from authentic luciferase and that its activity depends upon a noncovalently bound small molecule, probably corresponding to the absorption at 400 nm. Oxidized flavin will not substitute for this molecule; knowledge concerning its structure and origin should be helpful in understanding the species involved in bioluminescence initiated by FMNH2.

Acknowledgments—We are indebted to Professor Vincent Massey for suggesting the use of iso-FMN and to him and Dr. W. Föry for providing us with the compounds used. We are equally grateful to Dr. A. Eberhard for his interest, assistance, and advice with these experiments.

REFERENCES

