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BACTERIAL LUCIFERASE: BIOLUMINESCENCE EMISSION USING LUMAZINES AS SUBSTRATES

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Introduction

In the bacterial luciferase reaction reduced flavin mononucleotide ($FMNH_2$) serves as a reactant to form the flavin-4a-hydroperoxide [1,2], which, in turn, reacts with a long-chain aldehyde to yield the excited state [3]. Luciferase can use various flavin analogs in the reaction; such analogs may have effects on the spectrum, kinetics and yield of light emission [4,5,6]. However, no molecules other than flavin derivatives have been found to catalyze the emission of light. We report here that the reduced forms of tetrahydro-FMN (4H-FMN), which is structurally analogous to FMN but chemically a lumazine (see structures in scheme 1), and lumazine 8-ribityl phosphate, are also capable of acting as substrates in the light emitting reaction.

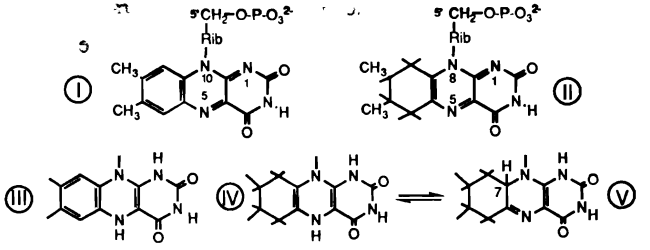
Results and Discussion

There is a major difference between reduced flavin, which is the 1,5-dihydro-tautomer (Scheme 1, III), and the products obtained upon 2 e- reduction of 8-substituted lumazines (IV, V). With the latter the 7,8-tautomer (V) is the more stable form thermodynamically [7], but luciferase from *Vibrio harveyi* evidently binds both, since the protein fraction after G-25 gel filtration has spectral characteristics indicative of a mixture of the two (Figure 1, curve A). Upon standing at 4 °C (and final warming at 25 °C), the spectrum changes to that shown in curve B, with isosbestic points at 358 and 505 nm. Further spectral changes occur upon treatment of the sample with 2% sodium dodecyl sulfate (curve C).
The existence of a luciferase-bound hydroperoxy intermediate is indicated by the fact that bioluminescence occurs upon the addition of long chain aldehyde to the protein fraction

from gel filtration. Its spectral identity is not known, but only the 5,8 dihydro form (IV) would be expected to form such a hydroperoxide, and it would be expected to be metastable [7].

Light emission can also be obtained by initiating the reaction with 4H-$FMNH_2$ in the presence of aldehyde. Its intensity is about 10% that observed with $FMNH_2$, and the emission maximum is shifted slightly to the blue (Figure 2). A dependence of the emission intensity on the 4H- $FMNH_2$ concentration yields an apparent Km of 8 μM which compares to 0.3 μM for FMNH2. A similar response was obtained with reduced lumazine 8-ribityl phosphate.

These results indicate that 4H-$FMNH_2$ can indeed replace the normal substrate of the *V. harveyi* luciferase reaction, and that the isoalloxazine ring system is not obligatory for the chemistry of the steps leading to light emission. With regard to its oxygen reactivity, the dihydrolumazine can be assumed to be similar to that of reduced flavin, and indeed the corresponding tetrahydropteridines have been shown to form hydroperoxides which are necessary intermediates during the hydroxylation of aromatic amino acids catalyzed by enzymes such a phenylalanine hydroxylase. We thus assume that the 5,8-dihydrolumazine chromophore bound to luciferase forms a hydroperoxide, which can react with long chain aldehyde in a way similar to that occurring in the normal reaction (Scheme 2). The primary function of the flavin/lumazine might thus be to provide a hydroperoxide of appropriate reactivity.



Scheme 1. Structures of FMN (I); tetrahydro-FMN (II); 1,5-dihydro-FMN (III); 5,8-dihydro-8-R-lumazine, obtained by the 2e- reduction of tetrahydro-FMN (IV), and the corresponding, tautomeric 7,8-dihydro form (V).

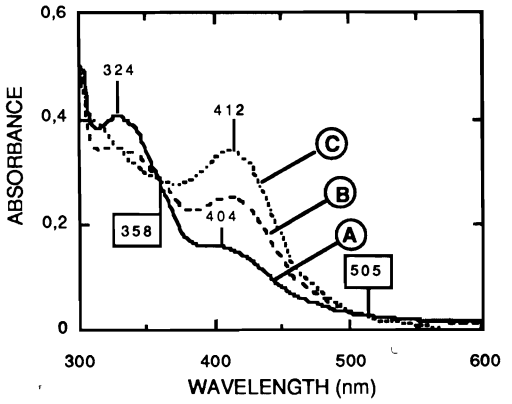


Figure 1. Isolation of luciferase-bound 4H-FMN species.

Bacterial luciferase (*Vibrio harveyi*, strain M17; 0.4 mL of a 100 μM solution) was mixed with 4H-FMN (0.25 mL of a 4.5 mM solution) and reduced with dithionite. The mixture was passed through a G-25 column (void volume 5 mL, 4° C) and the protein fraction was collected. Its spectrum is shown by curve A. Spectral changes occur spontaneously after isolation of this species, with isosbestic points at 358 and 505 nm. The final spectrum is represented by trace B, which was recorded after warming the sample to 25° C and recooling to 4° C. Spectrum C was obtained after treatment of this sample with 2% sodium dodecylsulfate and removal of the precipitate by centrifugation.

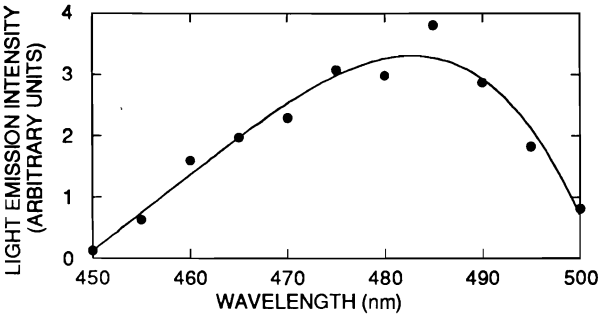
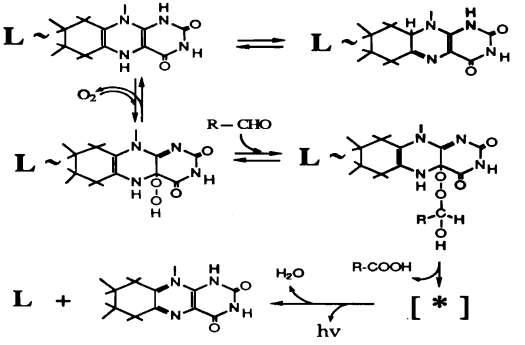


Figure 2. Bioluminescence emission spectrum with reduced 4H-FMN as the substrate. 4H-FMN in 0.5 mL 350 mM potassium phosphate, pH 7 was reduced with a dithionite solution in the presence of luciferase. Light emission was initiated by injection of 1 mL of a 0.01% decanal suspension in the same buffer and measured with a Perkin Elmer MPF-4

fluorimeter, with no light excitation, varying the emission monochromator. No correction for photometer response was carried out. The initial maximum light intensities obtained from single experiments are plotted.



Scheme 2. Postulated reaction sequence of the light emitting system with 4H-FMNH2. Note that luciferase is assumed to bind and stabilize preferentially the 5,8 -dihydro- tautomer of tetrahydro-FMNH2. The further sequence is similar to that proposed for FMNH2 [6].

Acknowledgements

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References:

1. Hastings, J. W., Q.H. Gibson. 1963. J. Biol. Chem. *238*, 2537-2554.

2. Hastings, J. W., C.Balny., C.H..LePeuch, P. Douzou. 1973. Proc. Natl. Acad. Sci. U.S.A. *70*, 3468-3472.

3. Kurfürst, M., S. Ghisla and J.W. Hastings. 1984. Proc. Natl. Acad. Sci. USA *81*, 1990-1994

4. Mitchell, G., J.W. Hastings. 1969. J. Biol. Chem. *244*, 2572-2576.

5. Kurfürst, M.P. Macheroux, S.Ghisla, J.W. Hastings. 1989. Eur. J. Biochem. *181*, 453-457.

6. Eckstein, J., S.Ghisla, J.W. Hastings 1993. Biochemistry *32*, 404-411.

7. Harzer, G., S. Ghisla. 1979. In: Chemistry and Biology of Pteridines, (Kisliuk, R. and Brown, G. eds.). Elsevier North Holland, Inc., pp. 37-42.