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AN EFFICIENT BACTERIAL BIOLUMINESCENCE WITH REDUCED LUMICHROME

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SUMMARY

Photoreduced lumichrome reacts *in vitro* with oxygen in the presence of tetradecanal and a specially purified sample of bacterial luciferase selected for minimum fluorescence at 490 nm upon 350 nm excitation from *Vibrio harveyi* yielding a rapidly decaying bioluminescence with an emission maximum at 476 nm. This emission is spectrally similar to the fluorescence spectrum of oxidized lumichrome. Less purified luciferase samples possessing appreciable fluorescence in the 490 nm region invariably gave bioluminescence at 490 nm. These results are interpreted in terms of an energy transfer mechanism.

Bacterial bioluminescence in vitro is commonly supposed to be the oxidation of $FMNH_2$ (reduced flavin mononucleotide) in the presence of long chain aldehyde and bacterial luciferase to yield light in the blue green region (1). The in vitro reaction has been shown to proceed with a number of chemically substituted flavins (2,3) and to produce light in different spectral regions with these. There is however up to now a strong belief that the in vitro reaction requires $FMNH_2$ and there has been a vigorous controversy within the literature (4-6) as to the stoichiometry with respect to $FMNH_2$.

We wish to report that the reaction can also proceed with the alloxazine derivative, reduced lumichrome, and it yields an emission clearly distinguishable from that obtained with the normally employed iso-alloxazine, $FMNH_2$. The reduced lumichrome induced bioluminescence is relatively efficient, yielding about 20% of the $FMNH_2$ bioluminescence under similar conditions.

MATERIALS AND METHODS

Bacterial luciferase was obtained from extracts of *Vibrio* (7) (formerly *Beneckea*) *harveyi* strain 392 (8) also known as "MAV". It was purified by a

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previously published method (4) with an additional ion exchange chromatography step to minimize the presence of 490 nm fluorophore. Subsequent to salt gradient elution on A50-Sephadex (8) the luciferase was concentrated by ultrafiltration (Amicon, PM30 membrane) and rediluted with distilled water so that the final phosphate concentration in the buffer was 0.15 M. This solution (about 500 mg protein) was loaded on to a column (2 x 20 cm) of DEAE-Sepharose (Pharmacia) pre-equilibrated with 0.15 M phosphate, 5°, pH 7.0 and eluted (60 ml hr^{-1}) with 0.175 M phosphate, pH 7.0, 4 liters. All buffers contained 5 mM 2-mercaptoethanol. All other chemicals were best commercial grades and used as supplied.

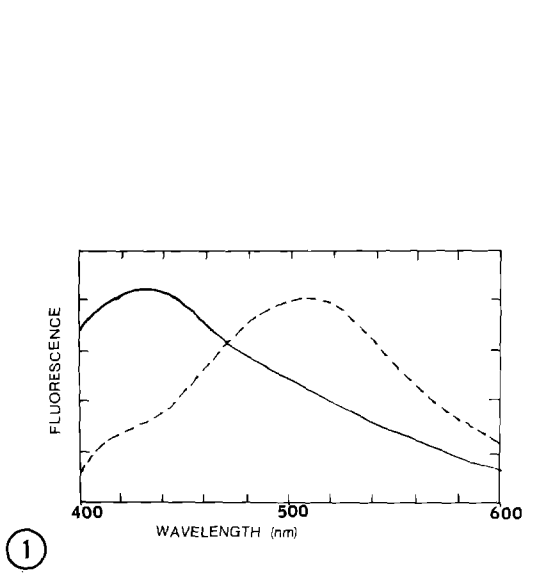
Reduced lumichrome was prepared by exposing lumichrome (80 to 160 μM) in pH 7.0 phosphate buffer containing 20 mM EDTA, to the focussed, unfiltered beam from a 150 watt Xenon lamp, for a period of 1 minute, by which time photoreduction was complete. Lumichrome concentrations were obtained by absorbance measurements assuming a molar absorbancy of 6000 at 342 nm in water (9).

Bioluminescence and fluorescence spectra were obtained using a computer controlled fluorimeter. In the bioluminescence mode no excitation light was present and the spectrum corrected for bioluminescence decay by means of a reference photomultiplier which measured the entire (unmonochromatized) emission spectrum. All spectra shown have been corrected for instrument response by calibration relative to an NBS Standard Lamp (10).

RESULTS AND DISCUSSION

The fluorescence of a protein sample A purified as described above and possessing an absorbance ratio at 280 nm to that at 450 nm, i.e., A_{280}/A_{450} = 600, is shown in Figure 1, solid line. The fluorescence excited at 350 nm of a less highly purified sample B prepared using the standard method (5), is shown in Figure 1, dashed line ($A_{280}/A_{450} \sim 250$). It is clear that B has the bulk of its fluorescence at 490 nm region, whereas A has little fluorescence at 490 nm and shows a maximum emission in the vicinity of 430 nm.

In Figure 2, upper panel, the bioluminescence due to the reaction of reduced lumichrome, 40 μM, in aerated pH 7.0 buffer at 0° with oxygen in the presence of 50 μM tetradecanal and 180 μM A, is shown. The fluorescence of oxidized lumichrome 140 μM in a 2 mm path length cell excited at 350 nm, is shown in Figure 2, lower panel. These spectra are extremely similar, λ max = 476 nm for the bioluminescence, and 480 nm for the fluorescence, suggesting the bioluminescence emitter is oxidized lumichrome. This bioluminescence required aldehyde and decayed rapidly with a half-life of about 3 sec at 0°.



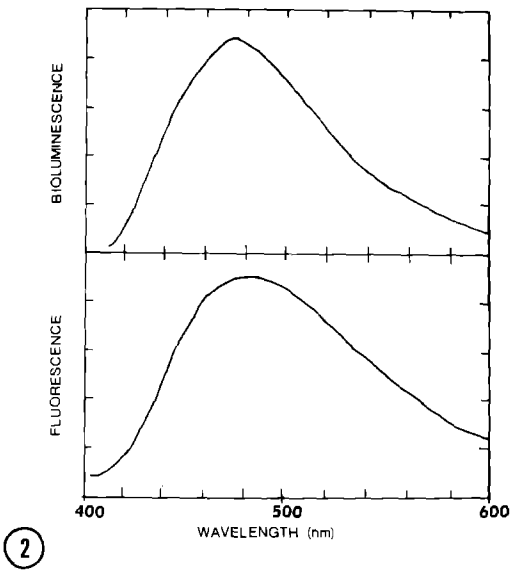


Figure 1. Relative fluorescence spectra of two differing luciferase samples. Solid line is sample A and dashed line is sample B.

Figure 2. Upper panel: relative bioluminescence spectrum of reaction initiated with reduced lumichrome using luciferase sample A. Lower panel: relative fluorescence of oxidized lumichrome.

The bioluminescence obtained using luciferase sample B at 200 μM in the presence of 50 μM tetradecanal in pH 7.0 buffer at 0° with 40 μM reduced lumichrome, is shown in Figure 3. This corrected bioluminescence shows a maximum emission near 490 nm, similar to the protein fluorescence shown in Figure 1, dashed line, and decayed with a half-life of about 5 seconds.

These results show clearly that:

i) the alloxazine reduced lumichrome is fully capable of driving the bioluminescence reaction, i.e., it can carry out the chemistry required to produce an electronically excited state. The maximum flavin contamination of sample A for which $A_{280}/A_{450} \approx 600$ is estimated to be 1.0 mole % of the protein, i.e., 1.3 μM which even if it were all converted to $FMNH_2$ by reduced lumichrome would be insufficient to account for the observed luminescence intensity, i.e. about 20% of that obtained using $FMNH_2$ under similar conditions, and comparable to the intensities observed with flavin analogs. An in vitro bioluminescence has been reported for reduced methyl red,

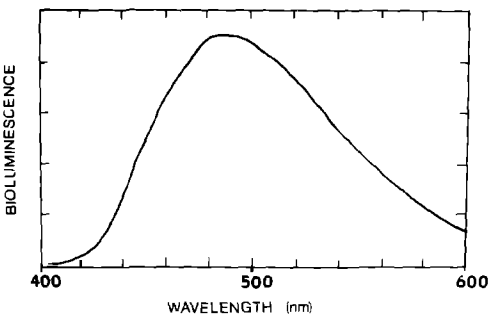


Figure 3. Relative bioluminescence obtained with reduced lumichrome and luciferase sample B.

showing < 1% of (2) and 5% of (11) the $FMNH_2$ reaction intensity in two separate studies. A weak bioluminescence, 10^{-4} of that observed with $FMNH_2$, has been reported to be obtained with H_2O_2 in the presence of FMN (12). The reduced lumichrome induced bioluminescence is distinguished from all other non-flavin systems in being relatively efficient. It is notable that lumichrome may be derived from riboflavin by photolysis in a number of solvents (14). It is also noteworthy that while lumichrome and flavin may be presumed to have similar electronic structures in their dihydro reduced forms they have clearly different spectral and redox properties.

ii) the bioluminescence emission observed is from a species present in the reaction mixture, lumichrome in the case of luciferase sample A and probably the species fluorescing at 490 nm present in sample B. A second possibility in the sample B case is reduced lumichrome reducing a flavin or flavoprotein in B producing $FMNH_2$ (13) which then effects the normal reaction producing 490 nm emission. The present results cannot distinguish between the emitter being present in B or being generated by reaction of something in B.

These results can be interpreted in terms of an energy transfer model where chemical reaction of reduced lumichrome leads to an excited state which can transfer either to oxidized lumichrome or probably the 490 nm fluorophore in B, the dominating emission being determined by relative concentration.

These results further show that the iso-alloxazines, flavins, are required neither for the chemical excitation process nor the emission process of in vitro bacterial bioluminescence. A more detailed report of the kinetics of bioluminescence of lumichrome will be given elsewhere.

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