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CHAPTER 58

The Bacterial Luciferase Neutral Flavin Radical: Identification and Catalytic Inactivity

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Introduction

The first step in the bioluminescent reaction catalyzed by bacterial luciferase involves the reaction of reduced flavin with molecular oxygen to form an oxygen-containing luciferase-bound 4a-peroxy flavin intermediate (2,4):

$$E\text{—}FMNH_2 + O_2 \rightarrow E\text{—}FMNH\text{—}OOH$$

When originally isolated and characterized, the peroxy intermediate was shown to exhibit absorption peaking at 373 nm, a shoulder at around 450 nm, tailing off at 500 nm, with no absorption above 520 nm.

In later work however, it was reported that an appreciable absorption in the 500–700 nm region occurred in such preparations, both in the presence and absence of long chain aldehyde (9,10). This observation prompted a series of speculations concerning the role of this new species and the mechanism of the luciferase reaction, (5,6,11,12). We were interested in the nature of this blue (red absorbing) intermediate, particularly with respect to whether or not it had some role in the sequence of processes involving oxygen activation, and population of the emitting excited state:

$$E\text{—}FMNH\text{—}OOH + RCHO \rightarrow (\ )^* \rightarrow h\nu + E\text{—}FMN_{ox} + RCOOH + H_2O$$

As described below, we have studied its formation and properties; the results show unambiguously that the species is a luciferase semiquinone neutral flavin radical and that it has no evident role in the bioluminescent reaction.

Results and Discussion

When the luciferase peroxide intermediate is prepared by reacting enzyme with FMN reduced photochemically in the presence of EDTA, followed by aeration directly in the cuvette, material with a spectrum such as that shown on Figure 1 is obtained. It is evident that a considerable absorption at >500 nm is present, which apparently corresponds to the red absorption reported previ-

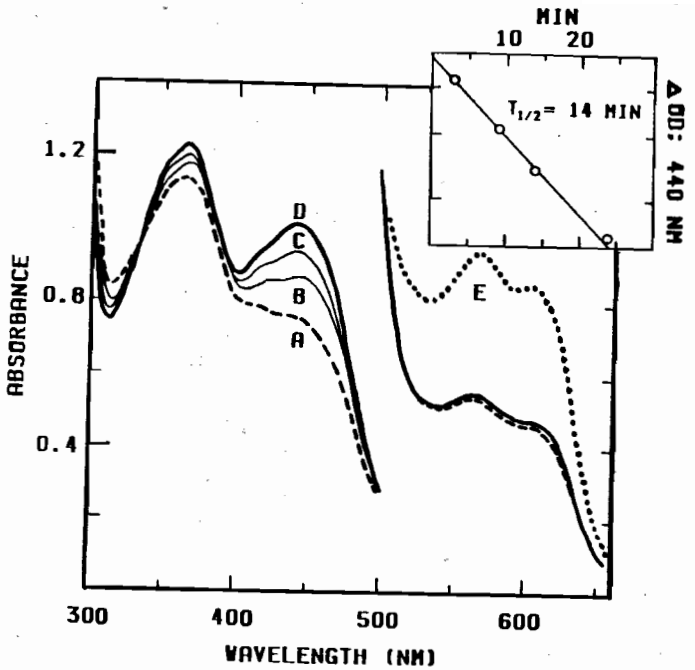


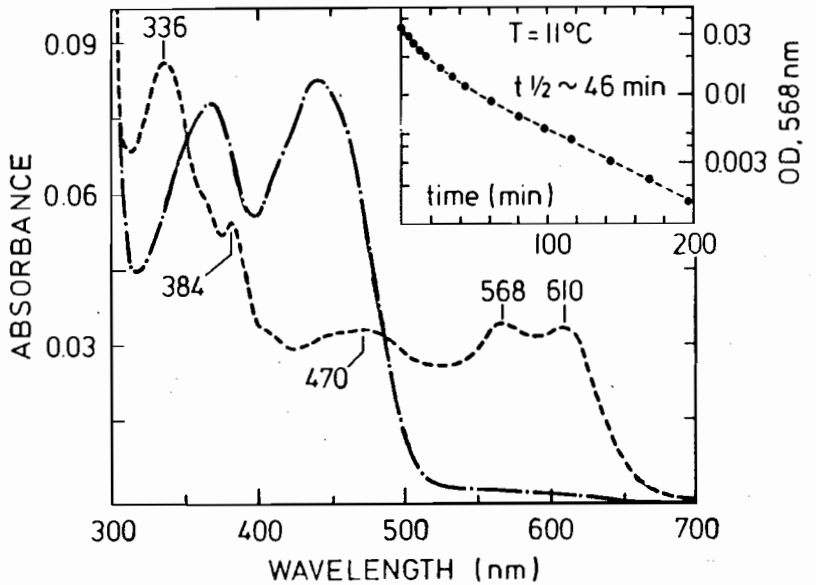
Figure 1. Absorption spectra of the products of the reaction of the luciferase-$FMNH_2$ complex with oxygen, and changes with time. *Beneckea harveyi*, (mutant strain M-17) luciferase (8×10^{-5} M), FMN(10^{-4} M), and EDTA (10^{-3} M), in phosphate buffer (0.2 M, pH 7) was made anaerobic by degassing, and then irradiated for 3 min to achieve full reduction of the flavin, as judged by the disappearance of the FMN_{ox} fluorescence. Oxygen was then admitted, and the first spectrum was recorded after 1 min (trace A). The absorption of the flavin peroxide peaks at about 370 nm, and its decay to oxidized FMN over the next 25 min (inset) was monitored by the increase at 440 nm (traces B, C, and D). The absorption of the blue species in the 500–700 nm range (scale at right, one division =0.05) was already present at the time trace A was recorded, and remained essentially constant during the decay of the peroxide. Repetition of the reduction-oxidation procedure as described resulted in an even greater amount of the blue species (trace E). Inset: ordinate, O.D. at 440 nm, plotted on a log scale; abscissa, time.

ously. In addition, there is absorption in the 300 to 500 nm range, this being identified with the luciferase flavin peroxide species ($_{max}$ =370 nm) previously reported. At 2°, the absorption in the 440 nm region increased ($t_{1/2}$ for increase to final value, 14 min), indicative of the decay of the peroxy flavin to form oxidized FMN (1). During this period, the absorption at 600 nm did not decay; in fact it increased slightly, so that after 26 min the OD at 560 nm was about 0.13. Subsequent to this, the photoreduction-oxidation cycle was repeated; this resulted in the formation of even more radical (Figure 1, trace E).

This result clearly differs from previous reports in two respects. In the earlier reports, there was no absorption in the 500 to 700 nm range, and the peroxide intermediate had a longer life time (40 to 50 min) at 2° (1,4). But such a behavior was indeed duplicated in the course of the present experiment by loading a dithionite (or photo-) reduced FMN-luciferase complex directly on a Sephadex G-25 column, and allowing oxidation to occur during separation (3). In this way, the luciferase-bound reduced flavin encountered oxygen only after its separation from small molecules, notably oxidized flavins, and no blue species was generated. When a similar preparation was allowed to react with molecular oxygen in the test tube for approximately 3 min prior to application to the column, a preparation was obtained with an absorption similar to that shown in Figure 1.

Similar amounts of the blue species were obtained with preparations reduced by dithionite, followed by aeration. When a sample prepared by this method was aged at 2° (thereby allowing the peroxide to decay), and then chromatographed on a short Sephadex G-25 column, a preparation was obtained containing essentially homogeneous blue species. Its spectrum (Figure 2) has maxima at 610, 568, 470, 384, and 336 nm, and is very similar to the spectra of neutral (blue) flavin radicals in apolar solvent (8), and in particular to the spectrum of the radical of *Azotobacter vinelandii* flavodoxin (7). From this and

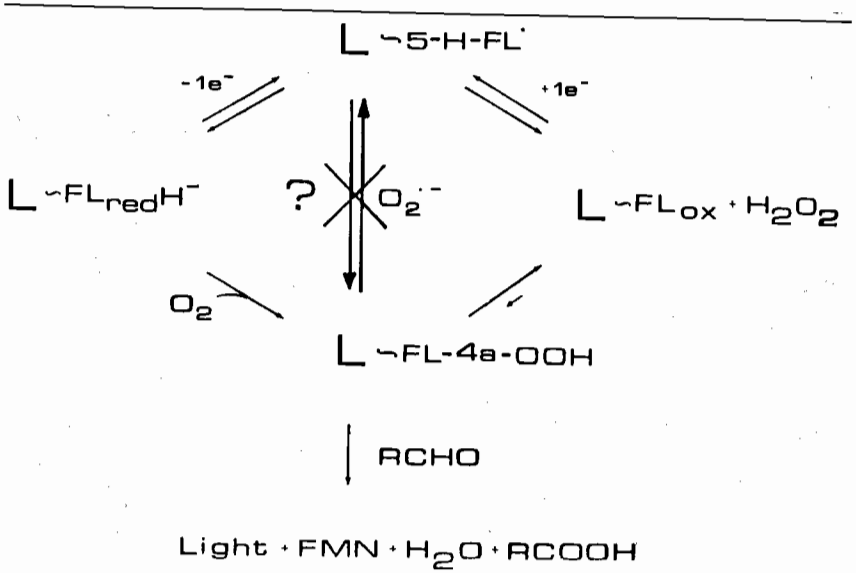
Figure 2. Absorption spectrum of the luciferase neutral (blue) radical and its decay product, enzyme-FMN_{ox}. The radical was prepared as follows: FMN, 2×10^{-4} M, in 0.5 ml 0.4 M phosphate buffer, pH 7 was reduced at 0° in the presence of 8×10^{-5} M luciferase with a small excess of dithionite in the same buffer. The mixture was then oxygenated, and the absorbance at 600 nm was measured. The formation of some additional absorbance at 600 nm occurred over the next 20 minutes, at which time the sample was chromatographed on a Sephadex G-25 column (void volume 4.5 ml) equilibrated with the same buffer. The protein fraction was collected, and its spectrum recorded immediately (---). (–·–·–) shows the spectrum of the same sample at the end of the decay process, after approximately 12 hr. The inset shows the time course of this decay 11°.

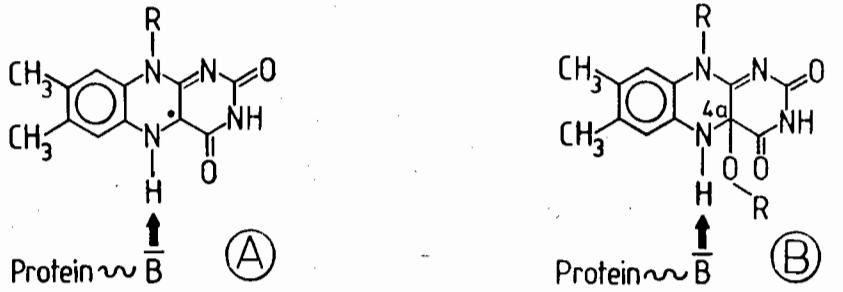


the results described below it was deduced that the blue species is a luciferase semiquinone radical. The radical nature of the blue species is confirmed by the finding of an essentially quantitative ESR radical signal, which decreases concomitantly with the disappearance of the 600 nm absorption. An identical blue radical can be generated by titration of the FMN_{ox}-luciferase complex with dithionite, by conproportionation of equimolar concentrations of FMN_{ox}, and FMN_{red} in the presence of luciferase, and also, presumably, as in preparations of Figures 1 and 2, by 1-e^- oxidation of the FMN_{red}-luciferase complex (Scheme 1).

The radical species is metastable, and decays isobestically to yield FMN_{ox} (Figure 2), the rate of the process being biphasic (Figure 2, inset), and highly temperature-dependent (Arrhenius activation energy, 40 kcal/Mole). Addition of decanal (a substrate in the luminescence reaction, reacting with the peroxide) to the radical generates no light emission. On the contrary, the stability of the radical is increased approximately threefold; the activation energy of the decay, however, remains the same. The high activation energy suggests that a large conformational change in the luciferase is involved in the decay of the flavin radical. The luciferase resulting from this decay possesses full activity.

Scheme 1. Proposed modes of formation and decay of the luciferase neutral (blue) radical. The reaction of the luciferase-$FMNH_2$ complex with oxygen to form the peroxide, and the reaction of the latter with long-chain aldehyde represent the catalytic pathway. $1e^-$ oxidation of the luciferase-reduced FMN complex (by, e.g., dithionite, EDTA-hv, or by excess FMN_{ox}) can form the radical (upper structure). It can similarly be formed by $1e^-$ reduction of the luciferase-FMN_{ox} complex. A further way of generation can consist of the enzyme binding preferentially HFMN$^{\cdot}$, which is formed by conproportionation of the oxidized and reduced FMN species. Direct interconversion of the luciferase peroxide and the radical does not appear to occur, at least not on a rapid time scale relevant for catalysis.





Scheme 2. Proposed mode of stabilization of the luciferase neutral radical, and luciferase-flavin 4a-intermediates. Structure (B) shows the hydrogen bridge which serves in the stabilization (i.e., prevention of elimination of R-O-H) of the peroxide, and hydroxy intermediates. The same interaction causes a stabilization of a neutral (blue) radical (A).

No luciferase radical is formed upon the decay of pure peroxyflavin, the latter prepared as described above. It is thus apparent that the blue neutral luciferase radical is not being formed from the peroxy species, and is not in a (relevantly rapid) equilibrium with it (Scheme 1). In fact, if such an equilibrium did exist, superoxide dismutase would effect it, this in contrast to the experimental findings, which show that superoxide dismutase or catalase have no effect on the decay rates of either the peroxide or the radical.

Conclusions

The mode of formation and decay of the luciferase blue radical, as well as its unusual stability, precluded any role of this species in the light-emitting catalysis. Its high stability however cannot be fortuitous. A comparison of the structure of such a radical [Scheme 2, structure (A)], with that of the transient species occurring during catalysis, i.e., the 4a-peroxide, and the 4a-hydroxy-FMN derivatives [Scheme 2, Structure (B)], indicate that in both cases the same mechanism of stabilization may be operative. This must consist of a strong interaction (hydrogen bridge) between the flavin (5) nitrogen, and a protein function. Thus, the occurrence of the blue radical is interpreted as reflecting a key property of the luciferase active center.

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