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Articles

Bioluminescence Emission from the Reaction of Luciferase–Flavin Mononucleotide Radical with $O_2^{-}\cdot$†

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ABSTRACT: The blue neutral luciferase flavin radical has been shown not to be in a catalytically significant equilibrium with species leading to emission of light [Kurfürst, M., Ghisla, S., Presswood, R., & Hastings, J. W. (1982) *Eur. J. Biochem. 123*, 355–361]. It is shown here that this radical can nevertheless react with $O_2^{-}\cdot$ to form a species that is competent in light emission. From its properties, the species formed is deduced to be luciferase–FMNH 4a-hydroperoxide, a key intermediate in the normal luciferase reaction. Although it is concluded that this intermediate can undergo a reversible homolytic dissociation to yield free superoxide and the corresponding luciferase radical, the slowness of these steps precludes a catalytic significance for these pathways in the normal bioluminescent reaction.

The enzyme bacterial luciferase catalyzes the oxidation of reduced FMN[1] and of a long-chain aldehyde with molecular oxygen to yield light, oxidized FMN, and the corresponding carboxylic acid (Ziegler & Baldwin, 1981; Hastings & Wilson, 1976). Catalysis has been shown to proceed via an intermediate, the luciferase-bound FMN 4a-hydroperoxide (Hastings et al., 1973; Tu, 1982), but further details of the mechanism have not yet been elucidated. It is likely that this peroxide is the species that oxidizes the aldehyde, thus forming a FMNH-4a-OH pseudobase, which is postulated to serve as the emitter (Hastings & Nealson, 1977; Kemal & Bruice, 1977; Hastings et al., 1981). We have recently shown that a blue species, which had previously been proposed to play a role in catalysis (Presswood & Hastings, 1979; Kosower, 1980), is in fact a very stable luciferase-bound neutral FMN radical and not in a relevant equilibrium with the species in the catalytic pathway (Kurfürst et al., 1982). This radical is not formed in significant quantities from homolytic fragmentation of the luciferase-bound FMN hydroperoxide; it is of importance, however, to determine if the putative products of such a homolysis can recombine to yield catalytically viable flavin peroxide:

$$\text{E–FMNH}\cdot + O_2^{-}\cdot + H^+ \rightarrow \text{E–FMNH-OOH}$$

Furthermore, it is of interest to determine whether the radical recombination as such might generate excited states or whether light is being produced subsequent to this process.

In this context, it should be noted that Nanni et al. (1981) have recently succeeded in forming flavin 4a-peroxides in a chemical model system starting from N^5-blocked flavin radicals and superoxide. In addition, using the pulse radiolysis technique, Anderson (1981) has shown that free flavin radicals combine with $O_2^{-}\cdot$ to yield a metastable species with an absorption in the 360–380-nm region compatible with that of known flavin 4a-OR derivatives. Catalytically viable luciferase flavin 4a-hydroperoxides have been obtained also from luciferase, FMN, and H_2O_2 (Watanabe & Nakamura, 1976; Hastings et al., 1979), i.e., by reversal of the reaction with the products of heterolytic decay.

Materials and Methods

Luciferase was isolated from the luminous bacterium *Vibrio* (formerly *Beneckea*) *harveyi*, mutant strain M-17, and was purified and assayed according to Hastings et al. (1978); it was 95% pure according to slab sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Lämmli, 1970). Enzyme concentrations were determined with $E_{280} = 74\ \text{mM}^{-1}\ \text{cm}^{-1}$

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[1] Abbreviations: FMN and $FMNH_2$, oxidized and reduced flavin mononucleotide; L–FMNH·, luciferase-bound neutral flavin radical; L–FMNH-4a-OOH, luciferase-bound flavin peroxide; SOD, superoxide dismutase; Me_2SO, dimethyl sulfoxide.

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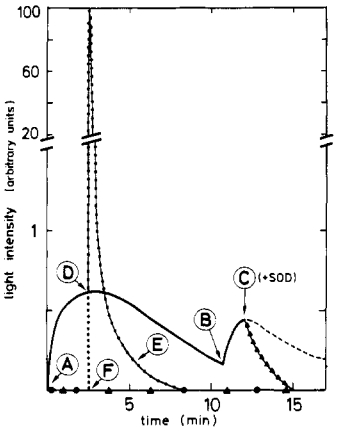


FIGURE 1: Light emission obtained from luciferase radical and superoxide. The reaction was initiated (at A) by addition of 0.05 unit of xanthine oxidase to an assay mixture consisting of 0.5 mL of 0.01 M phosphate buffer, pH 7.0, containing 10^{-4} M xanthine and 10^{-7} M luciferase radical. A further addition of xanthine after approximately 10 min (at B) caused a small secondary increase in the intensity. When SOD was present in the assay from the beginning, no light emission was detected (solid triangles), and the secondary addition of SOD (at C) caused the light emission to decay more rapidly. The secondary addition of decanal (3×10^{-4} M) to the assay mixture (at D) resulted in a large (~200×) increase in the light emission. However, the subsequent addition of SOD (at E) had no effect on the rate of light decay. The endogenous light emission was suppressed (large solid circles) when the incubation mixture contained 1.5×10^{-2} M hydroxylamine. However, subsequent addition of decanal in excess (at F) resulted in a light emission similar in intensity and decay kinetics to that observed in the absence of hydroxylamine.

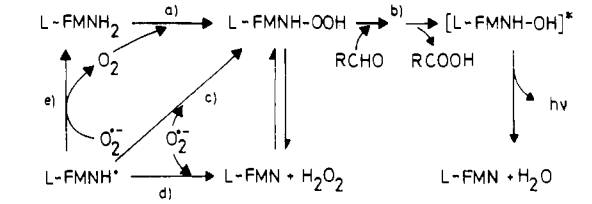
(Baldwin et al., 1975). Xanthine oxidase was from Boehringer, superoxide dismutase (lyophilized powder) from Sigma, hydroxylamine from Merck, and decanal from Aldrich. KO_2 (Fluka) solutions were prepared by the method of Valentine & Curtis (1975), i.e., by dissolving KO_2 in Me_2SO in the presence of 18-crown-6 ether (Aldrich). The xanthine solution (Merck) was prepared according to Avis et al. (1955).

The luciferase flavin hydroperoxide and the luciferase neutral flavin semiquinone were prepared and purified according to Kurfürst et al. (1982); the concentration of the latter was determined with the assumption of $E_{600} = 4800\ M^{-1}\ cm^{-1}$. Measurements and experiments were carried out at 23 °C. Absorption spectra and slow kinetic studies were carried out with a temperature-controlled Kontron-UVIKON 820 spectrophotometer and bioluminescence studies in a photometer (Mitchell & Hastings, 1971). Bioluminescence emission spectra were determined with a Perkin-Elmer MFP-44 fluorescence spectrofluorometer (slit, 6 nm; scan speed, 240 nm/min) without the exciting light and corrected for phototube sensitivity.

Results

Demonstration That Luciferase Radical $O_2^{-}\cdot$ and R—CH=O Yield Light. As previously described (Kurfürst et al., 1982), aerobic solutions of luciferase neutral radical decay slowly to yield FMN and luciferase. During this process no light emission is observed, either in the presence or in the absence of long-chain aldehyde, the normal luciferase substrate. The blue luciferase radical was also shown not to be in kinetically relevant equilibrium with the light-generating luciferase–FMNH 4a-hydroperoxide. However, we have now found (Figure 1, solid line) that superoxide ion ($O_2^{-}\cdot$) and the luciferase radical do react to generate a light-producing species, even in the absence of added aldehyde. [The origin of the "endogenous" light emission obtained in the absence of added aldehyde has never been fully explained. Some results suggested that the breakdown of the peroxide (see Scheme I) is accompanied by a low quantum yield (10^{-3}–10^{-4}) luminescence (Hastings et al., 1966); other results have been interpreted (Cormier et al., 1969) to mean that all light emission occurs via pathway b due to trace amounts of contaminating aldehyde.] The subsequent addition of decanal (at D) increased the intensity of the light emission about 200-fold, and the decay kinetics of the light emission were the same as for the normal luciferase reaction.

Scheme I



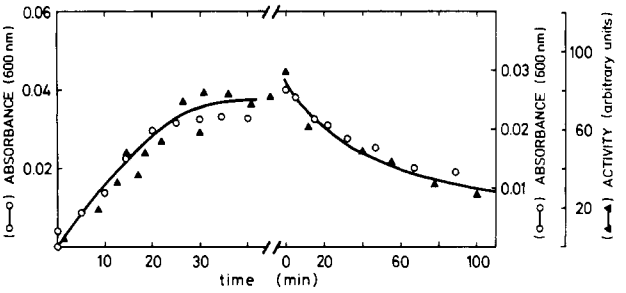


FIGURE 2: Luciferase radical concentration and light emission capability. The left-hand part of the figure shows the increase of luciferase neutral radical concentration (open circles) at 0 °C monitored by the absorbance at 600 nm ($E_{600} = 4800$), along with the light-emitting ability (solid triangles) of the sample measured with 10-μL aliquots. The right-hand part of the figure depicts similar measurements of the decay of the same preparation of luciferase radical at 10 °C after its purification on a Sephadex G-25 column. The radical was prepared by starting with 3 mL of 7×10^{-5} M luciferase in 0.35 M phosphate buffer, pH 7.0.

When SOD was present in the incubation mixture from the beginning, no light emission could be measured. But when SOD was added after aldehyde (Figure 1, E), no decrease in the luminescence occurred. However, the slower decay of the weak endogenous emission (no added aldehyde) was affected by SOD (Figure 1, C). This endogenous emission was quenched by hydroxylamine, indicating that it might be dependent, at least in part, upon contaminating aldehyde. The addition of decanal in excess, subsequent to hydroxylamine (Figure 1, F), resulted in light emission similar to that observed in the absence of the aldehyde trapping agent.

The same type of light emission occurred when the luciferase radical was reacted with different $O_2^{-}\cdot$-generating systems such as xanthine, acetaldehyde or purine with xanthine oxidase, or the system $O_2^{-}\cdot$ + 18-crown-6 (K^+, in Me_2SO; Arudi et al., 1981). The effects of systematic variation of the different reagents, trapping agents, and their sequence of addition, summarized in Table I, also demonstrate that L–FMNH·, $O_2^{-}\cdot$, and long-chain aldehyde are the requirements for light emission.

Figure 2 shows the correlation between the concentration of luciferase-bound flavin radical, measured by absorbance, and the luminescence capability of the system. The latter was estimated from small aliquots, whose light emission was determined after the addition of the xanthine/xanthine oxidase $O_2^{-}\cdot$-generating system. An increase in the absorbance at 600 nm (left side, Figure 2), corresponding to the slow formation of the luciferase radical, occurs over a period of 30 min after

Table I: Effect of Various Reagents and of Their Sequence of Addition on the Light Emission Obtained with Luciferase Neutral Semiquinone Radical (L-FMNH·)[a]

Sequence of Addition									
1	*hν*	2	*hν*	3	*hν*	4	*hν*	5	*hν*
L-FMNH·	–	xanth	–	Xa Ox	+	RCHO	++		
L-FMNH·	–	xanth	–	SOD	–	Xa Ox	–	RCHO	–
L-FMNH·	–	KO_2^{-}·	+	RCHO	++				
L-FMNH·	–	SOD	–	KO_2^{-}·	–	RCHO	–		
L-FMNH·	–	catalase	–	KO_2^{-}·	+	RCHO	++		
L-FMNH·	–	NH_2–OH (HCl)	–	xanth	–	Xa Ox	–	RCHO	++
xanth	–	Xa Ox	–	L-FMNH·	+	RCHO	++		
RCHO	–	L-FMNH·	–	KO_2^{+}·	++				
KO_2^{-}·	–	RCHO	–						
luciferase (L)	–	xanth	–	Xa Ox	–	RCHO	–		
luciferase (L)	–	FMN	–	Xa Ox	–	xanth	–	RCHO	–

[a] The final concentrations in 0.5 mL of 0.35 M aerobic phosphate buffer, pH 7.0, 20 °C, were as follows: L-FMNH· = 4×10^{-8} M; xanthine (xanth) = 2×10^{-4} M; xanthine oxidase (Xa Ox) = 0.05 unit; SOD = 200 units; KO_2^{-}· = 3×10^{-4} M; FMN = 1.5×10^{-5} M; luciferase = 2×10^{-6} M; hydroxylamine = 4×10^{-3} M; catalase = 0.1 mg. Light emission (*hν*): (–) no detectable light emission; (+) and (++) weak and 200 times brighter emissions, respectively. In the experiment of line 3, the preincubation of aldehyde with KO_2^{-}· caused no change in the intensity of the light emission.

the reaction of luciferase–$FMNH_2$ with O_2 (Kurfürst et al., 1982). The half-time for the decay of the species (right side, Figure 2) is of the order of 70 min at 10 °C; i.e., it is much longer than that of luciferase flavin peroxide at the same temperature (Tu, 1979).

Evidence for the Formation of Luciferase–FMNH Hydroperoxide from Luciferase Radical and O_2^{-}·. The observation that luciferase radical and O_2^{-}· plus decanal yield light suggests the intermediacy of the same luciferase–FMNH hydroperoxide that occurs in the normal catalytic process (Hastings & Nealson, 1977; Ziegler & Baldwin, 1981). The generation of the peroxide from the radical and O_2^{-}· can be inferred, but the low concentrations obtained precluded a direct spectroscopic observation of this species. In the case of its generation with the xanthine/xanthine oxidase system, the steady-state concentration of O_2^{-}· is probably low (Fridovich, 1970). From the experiments of Figure 1, and from similar ones initiated by addition of the O_2^{-}·-generating system after that of aldehyde, it is apparent that the appearance of light emission, i.e., the approach of the maximal steady-state level of light emission, occurs with a half-time of the order of 0.2–0.5 min. This indicates that the rate constant, k_1, for the formation of the peroxide from the radicals is comparatively small. This

$$\text{L–FMNH·} + O_2^{-}\cdot + H^{+} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{L–FMNH-4a-OOH}$$

should be considered in relation to the finding that the rate of homolytic decay of the peroxide, k_2, must also be very slow, with a half-time for the process exceeding several hours at 0 °C (Kurfürst et al., 1982).

At 0 °C, the addition of a molar excess (about 2-fold) of O_2^{-}· (18-crown-6, K^{+}, in Me_2SO) to the luciferase radical caused an immediate 20–30% drop in the 600-nm absorption and a concomitant increase in the 450-nm region (data not shown), indicating immediate formation of oxidized FMN. Spectral changes in the 360–380-nm region, where the luciferase hydroperoxide absorbs (Hastings et al., 1973), were dominated by the appearance of oxidized FMN and the development of some turbidity. This made it difficult to observe directly (by its absorption) the formation of the luciferase hydroperoxide. However, when the addition of O_2^{-}· (18-crown-6, K^{+}, in Me_2SO) to the radical was followed immediately by the addition of decanal, a strong emission of light was observed (Figure 3). The kinetics of this light emission are closely similar to those obtained starting with pure luciferase peroxide and decanal (Figure 3). Although the total

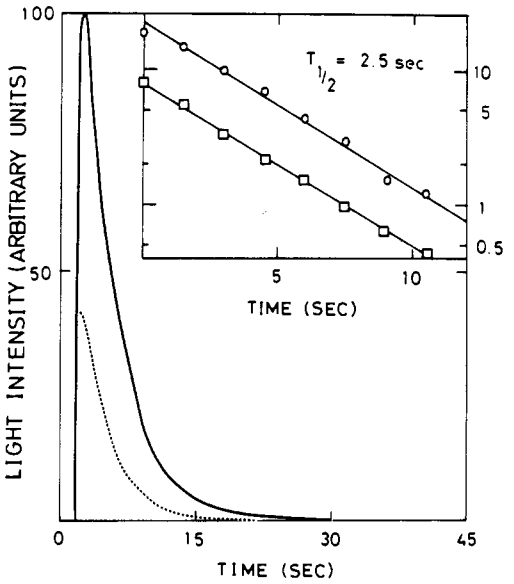


FIGURE 3: Comparison of light emission obtained from luciferase radical and superoxide with that obtained from luciferase peroxide. The solid line shows the time course of light emission obtained upon addition of 0.5 mL of decanal (final concentration 3×10^{-5} M) to 0.5 mL of luciferase flavin hydroperoxide (10^{-8} M) in 10^{-2} M phosphate buffer, pH 7.0. The dashed line is the luminescence obtained under the same conditions from the luciferase neutral flavin radical (2.5×10^{-8} M) reacted with superoxide (1.5×10^{-7} M final concentration, as the K^{+} salt in Me_2SO and in 3×10^{-7} M 18-crown-6 ether). The inset shows the semilogarithmic plots of the light emission decay of bioluminescence from the flavin hydroperoxide (circles) and that obtained from flavin radical and superoxide (squares).

amount of light produced from the luciferase radical was, on a molar basis, only 10–15% of that obtained from pure luciferase peroxide (as calculated from the area under the light emission traces of Figure 3), it can nevertheless be concluded that the generation of the excited emitter from luciferase neutral radical proceeds through the same pathway as in the normal bioluminescence reaction.

The formation of the luciferase hydroperoxide from the radical can be demonstrated more directly by chromatographic isolation of the species. Luciferase radical was incubated with the xanthine oxidase O_2^{-}·-generating system for 10 min at 2 °C and then chromatographed at the same temperature over a Sephadex G-25 column. Figure 4 shows that the amount of protein eluted in the different tubes correlates well with the capacity of the fractions to emit light simply upon mixing with

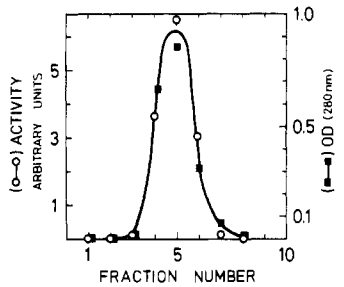


FIGURE 4: Gel filtration of L-FMNH· after incubation with xanthine and xanthine oxidase. A reaction mixture containing 0.5 mL of L-FMNH· (7 μM), 0.1 mL of a saturated xanthine solution, and 15 μL of a xanthine oxidase suspension (10 mg/mL) was incubated for 10 min at 0 °C. This mixture was then applied at 2 °C to a Sephadex G-25 column (void volume 5.5 mL; preequilibrated with 0.35 M phosphate buffer, pH 7.0) and eluted with the same buffer. Samples of 0.5 mL each were collected and kept at 0 °C. The light-emission capability (open circles) of samples of each was determined in 0.5 mL of 0.01 M phosphate buffer by the addition of 0.5 mL of 0.1% v/v decanal. The absorption of the fractions at 280 nm (solid squares) was measured after dilution with 0.3 mL of 0.35 M phosphate buffer.

decanal, and the kinetics of this light emission were the same as observed with authentic hydroperoxide. The decay of the activity of this preparation, which should reflect the decay of the luciferase hydroperoxide, occurs with a half-time of about 50 min at 0 °C, the same as that observed with authentic hydroperoxide (Becvar et al., 1978); This was measured by adding decanal to aliquots taken at different times from the preparation kept at 0 °, according to the method of Hastings & Gibson (1963).

Spectrum of Bioluminescence Induced by Reaction of Luciferase Radical with O_2^{-}·. As shown in Figure 1, a mixture of L-FMNH· and O_2^{-}· without added aldehyde results in a relatively steady level of light emission for a few minutes, allowing the wavelength dependence of the emission to be scanned repeatedly during this period. This emission spectrum, peaking at about 500 nm, is similar, but not identical, to that of the luciferase reaction itself (Figure 5). The slight difference is not due to the turbidity caused by the particles of undissolved xanthine; luciferase reactions with and without added xanthine showed no difference, and both reactions in the experiments of Figure 5 contained xanthine. Both spectra are from reactions without added aldehyde; there were no differences in the emission spectra with and without added aldehyde, as previously reported (Hastings et al., 1965a).

A possible explanation for the small difference in the emission spectra is that the emitters are different in the two reactions. Some possibilities, such as oxidized flavin (λ_{max} 530 nm) or the xanthine/xanthine oxidase system [λ_{max} 430 nm (Henry & Michelson, 1977)], may be excluded. However, it has recently been shown that, in some species of luminous bacteria (but up to now, not in *V. harveyi*), there occurs, in addition to luciferase, a second protein with a bound chromophore that can serve as a secondary emitter, probably by energy transfer (Koka & Lee, 1979; Leisman & Nealson, 1982). The present results might be explained if such a putative secondary emitter were involved in the normal reaction, but not in the luminescence resulting from the reaction of superoxide and the luciferase radical. Since the secondary emitter may enhance quantum yield (Gast & Lee, 1978), this could also account for the lower apparent yield from the luciferase radical. However, since pure *V. harveyi* luciferase emits light peaking at 495 nm, this explanation seems unlikely.

Discussion

The results of Figure 1 and those summarized in Table I show that the luciferase neutral radical, superoxide ion (O_2^{-}·), and long-chain aldehyde can combine to yield light. The requirement of all three reagents for significant light production indicates that the production of the excited state occurs via the same catalytic steps as in the normal reaction and that a luciferase–flavin 4a-hydroperoxide is required for the light-emission process. [However, see Matheson & Lee (1981).] Since catalase has no effect (Table I), it may be concluded that H_2O_2 and/or its recombination with L-FMN to form the luciferase peroxide does not play a role in this process. Of relevance in the context of the mechanism of production of luciferase excited states is also the observation that the recombination of the two radical species (L-FMNH· + O_2^{-}·) to form the peroxide (L-FMNH-OOH) does not itself produce luminescence other than that normally obtained from the peroxide in the absence of added aldehyde. The relation of the normal catalytic steps (a and b) to the radical recombination reactions is shown in Scheme I.

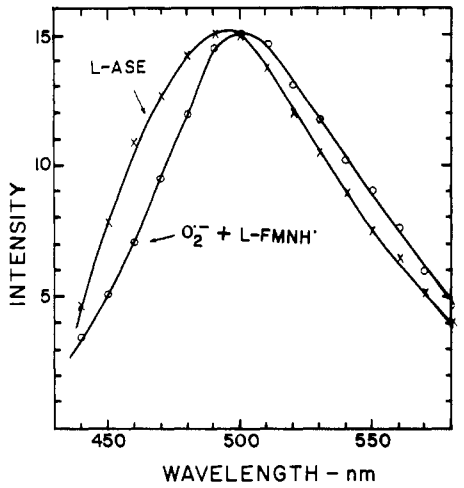


FIGURE 5: Emission spectra of bioluminescence of luciferase radical with O_2^{-}· (O) compared with the luciferase reaction (×). The luciferase neutral radical was mixed with xanthine and xanthine oxidase, as described in the legend of Figure 1. The luciferase reaction was carried out in the same buffer with a partially purified luciferase preparation containing reductase (Hastings et al., 1965b). The reaction contained 5×10^{-6} M FMN and was initiated with NADH (10^{-5} M).

Thus the superoxide-initiated reaction joins the catalytic path at the point of the luciferase hydroperoxide (step c) or, alternatively, e + a. A main reaction of O_2^{-}· and luciferase radical, however, appears to be the oxidation of the latter to FMN and, presumably, H_2O_2 (step d). A reduction of the radical by O_2^{-}· would form E-FMNH$_2$ and O_2 (step e), which are expected to react immediately to form the peroxide via step a. In our system, a kinetic differentiation between pathway c and pathway (e + a) is impossible as the rate of a is very fast (Hastings & Gibson, 1963). A differentiation between the two pathways is difficult in any case, since the exchange of electrons (redox equivalents) in such a caged complex is expected to be very fast and, moreover, the same thermodynamically favored product is formed in the two cases.

The dismutation of O_2^{-}· is known to lead to a weak light emission, maximal at 430 nm (Henry & Michelson, 1977), which is enhanced strongly in the presence of luminol and other compounds, such as *Pholas* luciferin. With our photometer and under our experimental conditions this light emission was not detectable (less than 10^{-3} of the emission with the radical; Table I) and thus cannot account for the observed phenomenon. Furthermore, the emission spectrum is very different from that observed. Also, such a light emission should be quenched immediately and completely by addition of SOD,

which did not occur in our experiments (Figure 1,C). The slow decay of luminescence after SOD addition can be attributed to previously formed luciferase peroxide; superoxide dismutase in this case stops the further production of O_2^{-}·.

The present results are in full agreement with the observed generation of flavin 4a-hydroperoxides in model chemical systems from the flavin radical and O_2^{-}· (Nanni et al., 1981; Anderson, 1981; Bruice, 1982). They are also consistent with the observations reported earlier that luciferase and FMN can react with H_2O_2 to yield enzyme-bound peroxide (Watanabe & Nakamura, 1976; Hastings et al., 1979). We have shown that recombination of radical species can lead to light emission but that it is not the recombination step as such that generates the excited states responsible for light emission. Nevertheless, the role of radicals and electron-transfer processes at some later step(s) in catalysis remains a mechanistic possibility for the population of the excited state (Kosower, 1980; Faulkner, 1978).

Registry No. Superoxide, 11062-77-4; FMNH·, 81138-29-6; FMNH-4a-OOH, 71368-40-6; luciferase, 9014-00-0.

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