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# PROPOSED MECHANISM FOR THE BACTERIAL BIOLUMINESCENCE REACTION INVOLVING A DIOXIRANE INTERMEDIATE

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We propose here a verifiable mechanism for the bacterial bioluminescence reaction involving a dioxirane intermediate. Participation of the dioxirane predicts either formation of an excited carbonyl, rather than the flavin, as the primary excited state in the reaction, or, through a CIEEL mechanism, the C4a hydroxyflavin or the chromophore of a secondary emitter protein could become excited. We propose energy transfer from the primary excited state to the C4a hydroxyflavin in the absence of the lumazine protein or the yellow fluorescence protein, while in the presence of either of the secondary emitter proteins, excitation energy would be transferred to the second protein-bound chromophore. The mechanism is similar to other currently discussed mechanisms, except in the final steps leading to the primary excited state. The mechanism is consistent with the known details of the reactions of dioxiranes and of flavins and with recent studies of the secondary emitter proteins and bacterial luciferases. 

Bacterial luciferase catalyzes the reaction of $FMNH_2$, $O_2$ and a long-chain aliphatic aldehyde to yield FMN, the carboxylic acid and blue-green light ($\lambda_{max}$ ~ 490 nm; 1). The en-

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + h\nu$$

zyme is formally a flavin monooxygenase, splitting $O_2$ to form a hydroxylated substrate and water. Bacterial luciferase is unique within the flavin monooxygenases, in that during the course of the reaction, an electronically excited intermediate is formed which is capable of emission of a photon of light. If other flavin monooxygenases emit light, they do so at an exceedingly low quantum yield, immediately suggesting a unique contribution from the enzyme in either the formation of the excited state or protection of the excited state from quenching.

It has been widely accepted for many years that in the *Vibrio harveyi* system, light emission comes from an enzyme-bound flavin (1-3). Mitchell and Hastings showed that the spectrum of the emission from reactions involving certain flavin analogs was altered, and Cline and Hastings showed with the *V. harveyi* system that mutations could lead to specific

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lesions in the protein causing an altered spectral distribution. The mutant AK-6, which is α113Asp→Asn (4), has a bioluminescence emission spectrum that is red-shifted by ca. 12 nm both *in vitro* and *in vivo*. These two observations demonstrate that the spectrum of the emitted light is a property of both the flavin and the luciferase, suggesting that emission is from an enzyme-bound flavin (1). The finding that the emission spectrum of AK-6 is red-shifted *in vivo* suggested that, at least in *V. harveyi*, light emission comes from a luciferase-bound flavin.

The situation with *V. fischeri*, *P. phosphoreum* and *P. leiognathi* appears to be different, in that the emission spectrum *in vivo* is usually significantly blue-shifted compared with the emission spectrum from the purified luciferase, except for the *V. fischeri* strain Y1, which emits yellow light due to the yellow-flourescence protein (5-7). Lee and his colleagues, working primarily with the luminescence systems of *V. fischeri* and *Photobacterium phosphoreum*, have demonstrated the existence in certain strains of a secondary emitter protein which can interact with the luciferase, accepting excitation energy from a luciferase-bound intermediate, to emit light at a different wavelength (8-10). The lumazine protein, which was purified and characterized by Lee, causes an apparent *blue-shift* in the emission maximum of the light emitted from reactions to which it is added, suggesting that emission *in vivo* is not from a luciferase-bound flavin, but rather from the secondary emitter, the lumazine protein.

The lumazine protein-mediated blue shift caused some concern, due to the apparently unfavorable transfer of energy from a lower to a higher energy state, and led to the suggestion that the primary excited state in the reaction might be something other than the flavin, such as a carbonyl, which could transfer to either the flavin or to the lumazine protein (1). Since that time, other authors have agreed with the possibility, but no verifiable mechanism has been suggested which incorporates the apparent necessity to form a more energetic primary excited state, prior to formation of the singlet excited state of the flavin.

***Intermediates in the Luciferase Reaction.*** The various intermediates in the reaction that have been detected and described are presented in Fig. 1, together with several possible intermediates that have not been clearly delineated. In the normal assay, the enzyme is incubated in a buffer containing aldehyde and dissolved $O_2$. The reaction is initiated by rapid injection of $FMNH_2$. The pathway that is usually discussed is given in the top line of Fig. 1, in which the first step is the equilibrium binding of $FMNH_2$ to the enzyme to form Intermediate I (11, 12). Intermediate I then reacts rapidly with $O_2$ to form intermediate II, the C4a peroxydihydroflavin intermediate that is the long-lived intermediate in the reaction (12, 13). Intermediate II can decay in a nonluminescent reaction to yield FMN and $H_2O_2$, or it can bind aldehyde to form Intermediate IIA. The lower series of reactions in Fig. 1 depict two possible intermediates resulting from binding of aldehyde to the free enzyme and to Intermediate I, to form Intermediate A (enzyme-aldehyde) and Intermediate IA (ternary complex of enzyme-aldehyde-$FMNH_2$). While these species have not been demonstrated, neither have they been ruled out, or even seriously discussed in the luciferase literature, and therefore should be considered as possible participants.

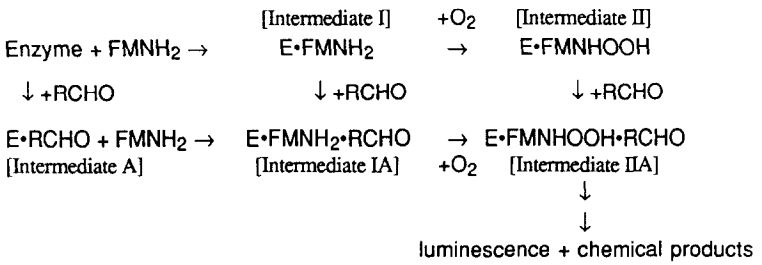
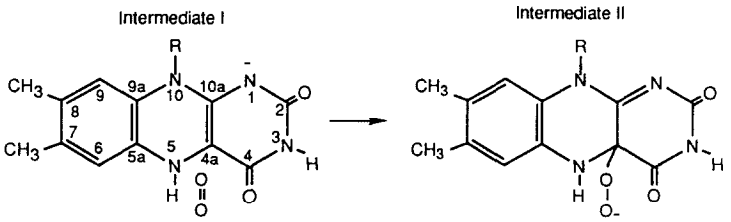


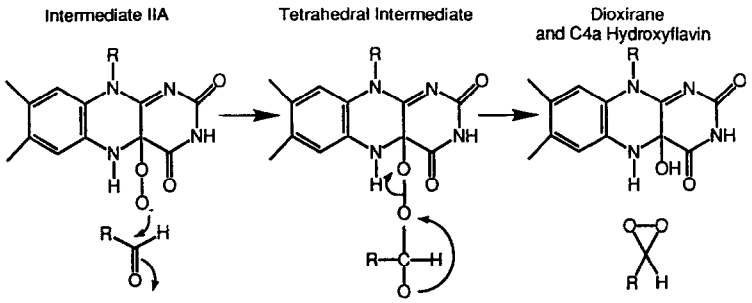

*Figure 1.* Reaction pathway of the bacterial luciferase-catalyzed bioluminescent flavin-mediated monooxygenation of an aliphatic aldehyde. This pathway depicts both well-documented intermediates (Intermediates I, II, and IIA) and possible intermediates (A and IA) which deserve further consideration.

**Proposed Mechanism.** In our mechanism, the first step involves reaction of diatomic oxygen with reduced FMN to form the C4a peroxydihydroflavin. In Scheme 1, N-1 of the luciferase-bound reduced FMN is the anion in accord with the findings of Vervoort et al. (14). The second step involves reaction of the peroxide with the carbonyl carbon of the aldehyde substrate to form the tetrahedral intermediate. These first two steps do not differ from other currently discussed mechanisms. In the third step, however, rather than removing the $\alpha$ proton from the tetrahedral intermediate to initiate a Baeyer-Villager rearrangement, we propose that the aldehydic oxygen attacks the peroxide linkage resulting in cleavage of the oxygen-oxygen bond to yield the flavin C4a hydroxide and the dioxirane (Scheme 2). The formation of the dioxirane is central to our proposal and there appears to be good chemical precedent for this alternative to the Baeyer-Villager rearrangement which leads directly to the carboxylic acid. Adam et al. have discussed a mechanism which is formally analogous to ours for the formation of a dioxirane intermediate during the ketone-catalyzed degradation of caroate ($KHSO_5$; 15).

There appear to be two potential chemiluminescent pathways for the breakdown of the dioxirane, both sufficiently energetic to populate an excited state yielding a photon in the blue. Adam and his coworkers suggest that the dioxirane could undergo homolytic cleavage of the oxygen-oxygen bond to yield the diradical, which should rearrange to form the carboxylic acid in either the triplet or singlet state (Scheme 3). This proposal has clear similarities to the degradation of dioxetanes (16). Even the triplet state should have sufficient energy for detection by means of enhanced chemiluminescence in the presence of a suitable fluorophore (15). The postulated C4a hydroxyflavin is such a fluorophore which could




Scheme 1

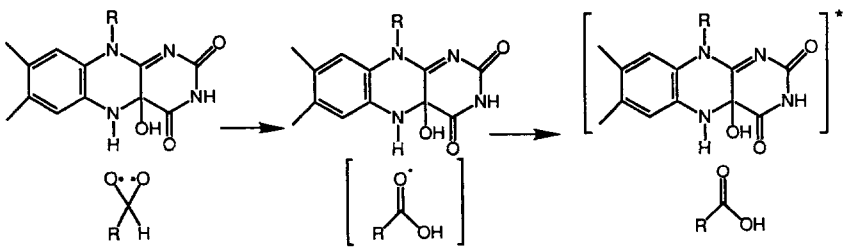



Scheme 2

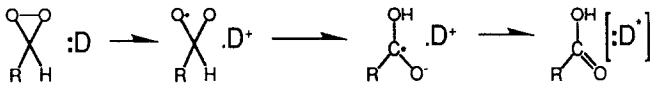
become excited by interaction with either the triplet or singlet product of the decay of the dioxirane. If the primary excited state in the reaction were the carboxylic acid, then the most likely emitter in the reaction catalyzed by pure luciferase would be the flavin. Addition of either the lumazine protein or the yellow fluorescence protein would supply an alternative fluorophore.

An alternative path to generation of an electronically excited fluorophore is the chemically induced electron exchange luminescence (CIEEL; 17) process in which the dioxirane receives an electron from a donor/fluorophore to yield a radical ion pair. Conversion of the dioxirane radical anion to the carboxyl radical and electron back transfer would yield the electronically excited fluorophore (Scheme 4). In the case of luciferase, we propose that the flavin hydroxide could play the role of the electron donor/fluorophore. In the presence of the lumazine protein or the yellow fluorescent protein, direct protein:protein interaction between the luciferase and the secondary emitter protein could result in participation of the chromophore of the secondary emitter protein in the CIEEL process with the luciferase-bound dioxirane.

This proposed mechanism incorporates a clear solution to the long-standing uncertainty regarding the apparent blue-shift in bioluminescence mediated through the lumazine protein (1). In fact, it was this enigma that lead to the proposal nearly ten years ago that the flavin might not be the primary excited state, but that there might be some other higher energy state that could excite either the flavin or the lumazine protein. Both the lumazine protein (10) and the yellow fluorescence protein (18) appear to interact directly with the luciferase and to



Scheme 3



Scheme 4

cause an acceleration in the luciferase-catalyzed reaction. This observation does not allow us to descriminate between the CIEEL mechanism and the direct transfer mechanism, since in the former, the secondary emitter protein would supply an alternative donor and therefore would be expected to effect a kinetic change, and in the latter, the direct protein:protein interaction could well contribute to the observed kinetic alterations. Furthermore, both mechanisms involve a comparable shift of an H· atom, such that distinguishing on the basis of isotope effects would be difficult. It is interesting to note that the expected isotope effect by either mechanism would be small, and indeed, we find a small effect of substitution of deuterium on the α carbon of the aldehyde on the rate of the bioluminescence reaction (Wilson Franscisco, unpublished). Finally, we have generated mutant luciferases by site directed mutagenesis that produce carboxylic acid product but with production of only very low levels of light (19, 20). These mutants could be explained on the basis of a mechanistic shift from formation of the dioxirane to degradation of the tetrahedral intermediate by the Baeyer-Villager reaction, a side reaction that one would expect for the wild-type enzyme as well. In the direct transfer mechanism, the chemical products are formed in the excited state prior to participation of the fluorophore. This fact argues, but not compellingly, for the direct transfer mechanism, if the dioxirane is formed during the nonluminescent reaction catalyzed by our mutant enzymes.

**Summary.** We propose here that the bacterial bioluminescence reaction proceeds through the tetrahedral intermediate to the formation of a dioxirane. The dioxirane could break down to form the carboxylic acid in an electronically excited state, or it could participate in a CIEEL reaction with either the luciferase-bound flavin or one of the secondary emitter proteins. Either pathway would be expected to yield luminescence, and the participation of the dioxirane would explain the failure to find luminescence from reactions catalyzed by other flavin monooxygenases.

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